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General Morphology and Stomatogenesis of Two Species of the Genus *Entodinium* (*Ciliophora, Entodiniomorphida*)

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Synopsis. In the two species studied of the genus *Entodinium* (*E. caudatum* and *E. longinucleatum*), the buccal infraciliature consists of only one polybrachykinety, on the left side of which can be found some kinetosomes with short cilia.

During the division of these ciliates, two oral primordia appear in the equatorial zone; both are independent entities, until they fuse to form the polybrachykinety of the opisthe.

Little is known about the morphogenesis of the entodiniomorphes ciliates, in spite of studies made by Dogiel (1927), Fernández-Galiano (1958, 1959, 1979); Noirot-Timothée (1960) and Wolska (1978 a, b). We have therefore made an attempt to study the formation of the buccal infraciliature during the process of division and have selected *E. caudatum* and *E. longinucleatum* for this aim since both species appear frequently in our samples.

Materials and Methods

The specimens of *Entodinium* studied were taken from the rumen and reticulum of cows slaughtered in several localities: Teruel, Alcorcón and Villaviciosa de Odón (Spain).

Having eliminated the plant remains by means of filtration, fixation was then achieved using formaldehyde (5%). The samples were then washed several times and kept in air-tight glass bottles at room temperature.

The pyridinated silver carbonate method (Fernández-Galiano 1976) was used for staining the specimens which made possible an accurate observation of the infraciliature and associated fibrillar structures in spite of the thick cuticle which protects the bodies of these ciliates.

Results

General Morphology

Both species of the genus *Entodinium* have a laterally compressed body which is divided into two zones: ectoplasm and endoplasm.

The ectoplasm contains the principal organelles: the semirigid pellicle, the nuclei (macronucleus and micronucleus), the contractile vacuole, and the infraciliature with its fibrillar systems. The endoplasm is formed by a central sack which is surrounded by the ectoplasm. It begins at the cytostome and ends at the cytoproct. It contains the digestive vacuoles, reserve granules, and cellular oganelles (Pl. I 1).

The buccal opening is situated in the anterior zone and is surrounded by the buccal infraciliature.

Entodinium longinucleatum has a barrel-shaped body rounded off at its posterior end.

The ciliate body is 54 μm long and 37 μm wide (in fixed specimens) (Pl. I 1 a).

Entodinium caudatum is more or less quadrangular in shape with three spines at the posterior end. The longest spine is on the dorsal part and the other two are on the ventral part of the body.

The body length is variable and ranges from 35 μm to 50 μm; its width is usually about 25 μm to 38 μm (Pl. I 1 b).

The Adoral Ciliary Zone

In both species, the adoral polybrachykinety consists of short parallel kineties and the kinetosomes of the adoral cilia which lie on rods. These rods, which have a constant orientation are dorsoventral on the right side and ventrodorsal on the left side. Thin cross connections were also observed in the adoral polybrachykinety (Pl. I 2).

The existence of a small group of characteristically disposed kinetosomes that give rise to the short and thick cilia protected by the internal and external adoral lips, was observed in the periphery of the adoral polybrachykinety, in the ventral zone of the ciliate (Pl. I 6).

For clarification purposes, we have divided this area into four parts: left ventral part, transversal part, left dorsal part, and intrainfundibullary part, respectively (Pl. I 3-5).

This ciliary zone does not fully surround the peristome; it begins on the left side of the body, continues to the ventral side and then passes on the right side towards the dorsal part of the ciliate where it turns left again towards the ventral side and finally enters the body through the infundibulum.

Division (Text-Fig. 7)

In the genus *Entodinium*, the division is apokinetal. The adoral polybrachykinety of the opisthe is formed "de novo", without any relationship with the kinetosomes of the adoral polybrachykinety of the parental cell.

The division begins when a fine line of kinetosomes arises on the right side of the ventral part in the middle zone of the body. Later, these kinetosomes are grouped together parallely in thin oblique striae (Pl. II 8, 9).

Simultaneously at the same level but on the dorsal left side another group of kinetosomes appears which are then reorganized into streaks.

The micronucleus then divides by a process of mitosis.

The new formed ventral right primordium stretches out towards the dorsal side of the body, at the same time, projecting itself towards the left side and increasing in width throughout the process. Meanwhile the dorsal left primordium moves towards the ventral side and finally joins the left part of the other primordium (Pl. II 10).

At this stage, the existence of kinetosomes, which later give rise to the short cilia, can be observed below the ventral primordium (Pl. II 11).

The fine line of striae that joined both primordia, now disappears and the dorsal left primordium remains in a oblique position to the anteroposterior axis of the ciliate (Pl. II 12, 13).

The micronucleus finishes dividing and the migration of one of the two micronuclei towards the opisthe now begins. At the same time, the division of the macronucleus commences by a process of elongation.

Finally, the dorsal extremes of both primordia join together to form the adoral polybrachykinety of the opisthe, whose striae have the same orientation as the proter.

Discussion

We have referred to the adoral ciliary band observed in *Entodinium* as adoral polybrachykinety, this term was first introduced by Fernández-Galiano (1979), to designate a great number of short, parallel kineties, closely grouped together. In 1932; Gelei and Se-

bestyen, named this structure "Sincilie", but this term tends to be confusing since Grain (1969) uses it to describe a different structure. The existence of polybrachykineties, is not exclusive to the subclass *Vestibuliferia* but it is characteristic of it.

The adoral polybrachykinety corresponds to the adoral (or buccal) ciliary zone described in other genera of entodiniomorphids such a *Polyplastron* (Fernández-Galiano 1958), *Eudiplodinium* (Noirot-Timothée 1960) and *Epidinium* (Noirot-Timothée 1960, Furness and Butler 1983).

Moreover, we have observed some perpendicullar fibers in the striae of the adoral polybrachykinety, so the band takes on a net-like appearance. These fibers are similar to those described by Noirot-Timothée (1960) in *Eudiplodinium medium medium*; she has called them "fibres interblepharoplastiques", that join the kinetosomes together, or they might be similar to the inter-kinetal microtubules described by Furness and Butler in *Epidinium caudatum* (1983).

Imai et al. (1983) describe two ciliary zones in the adoral polybrachykinety, the inner and the outer zones, which differ in arrangement and structure. In fact we can observe in their photomicrographies that the adoral ciliary zone presents two zones: the outer zone is made up by membranelle-like structures, that bear long cilia, and the inner zone, with shorter cilia and without unsystematic bundless. As we show in the present work it seems that there are not morphological differences in the infraciliary bases of the adoral polybrachykinety, so we can not distinguish the two zones mentioned above. This arrangement corresponds with the "syncil" defined by Grain (1962): "un syncil est un paquet de cilis, qui voisine avec d'autres paquets identiques dont il est séparé au niveau des cils; cette séparation n'existe pas au niveau des cinétosomes".

The kinetosomes observed in the periphery of the adoral polybrachykinety, in the right dorsal zone of the ciliate, seem to be homologous to those described by Coleman and Hall (1971) in *E. caudatum* in the specialized sheet-line extensions of the cell surface, and by Noirot-Timothée (1960) in the genus *Eudiplodinium* belonging to the same family. Besides, these kinetosomes have been observed in other species of the order *Entodiniomorphida* (Wolska 1978 a, b, Fernández-Galiano 1959) and might have a sensory function in relation to their external environment.

The adoral polybrachykinety is formed during cell division from a double primordium, like in the above mentioned genera, but with the difference that in those genera there is a third primordium which originates the anterior dorsal zone (Noirot-Timothée 1960). The plu-

rality of the primordia during the division of the ciliate, has been observed previously by other authors in different species (Schuberg 1891, Eberlein 1895, Günther 1899, Fernández-Galiano 1958, Noirot-Timothée 1960), but our study goes further and also illustrates the complete evolution of the new adoral polybrachykinety.

Contrary to Noirot-Timothée (1960), we observed that the orientation of the striae of the adoral polybrachykinety in the proter and in the opisthe is not inverted and we are inclined to think that this author observed the specimens in contract stages which would explain the inverted position of the adoral polybrachykinety.

Finally, the aparition of the kinetosomes corresponding to the short cilia during the process of the division has been observed for the first time.

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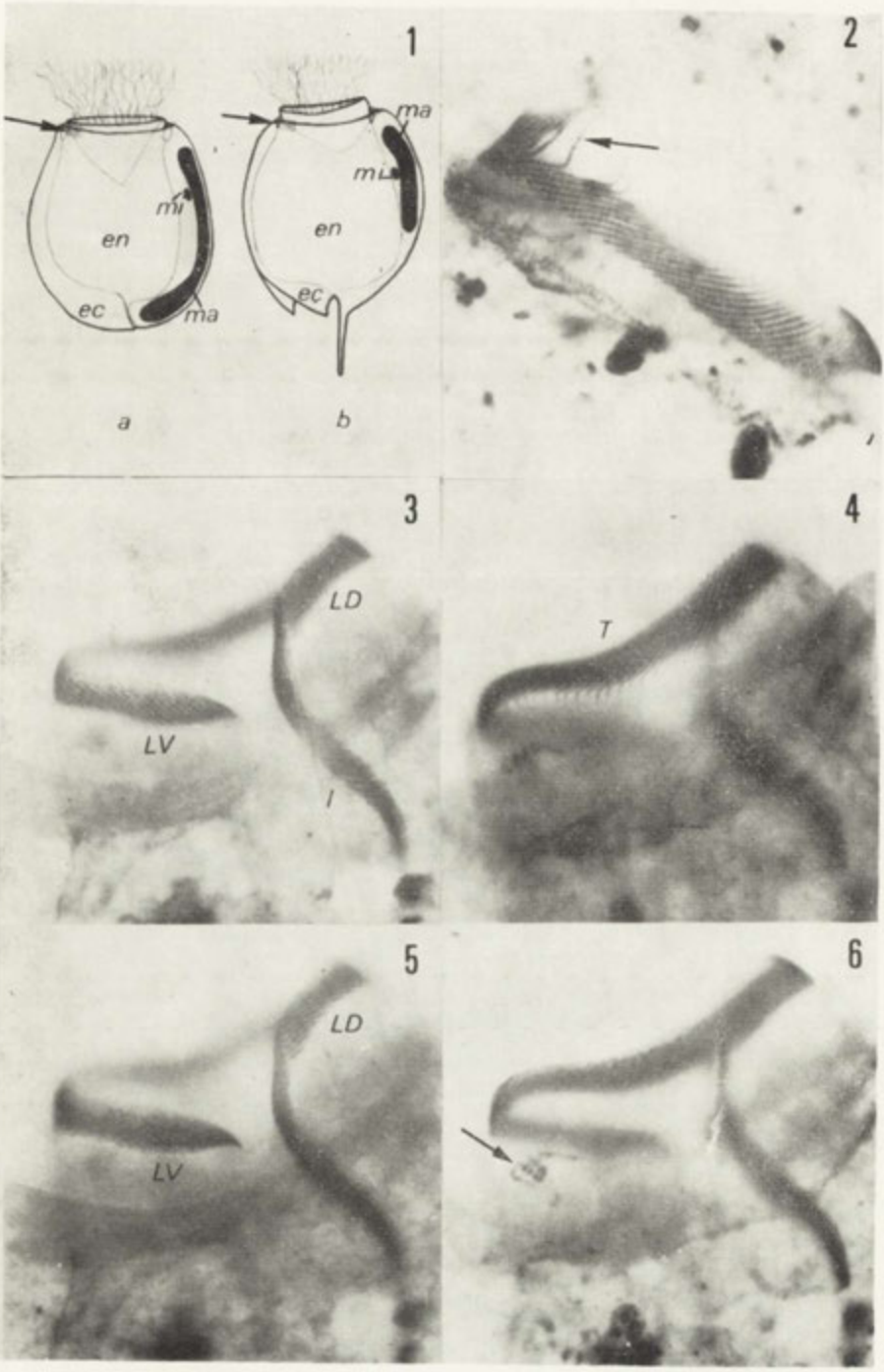
EXPLANATIONS OF PLATES I-III

1: General morphology of *Entodinium*. (a) *E. longinucleatum*, (b) *E. caudatum*. ma — macronucleus, mi — micronucleus, ec — ectoplasm, en — endoplasm. Arrow points sensory cilia
2: Detail of the adoral polybrachykinety of *E. caudatum*. Arrow points a kinetosome
3-5: Adoral polybrachykinety of a specimen of *E. longinucleatum*. LV — left ventral part, T — transversal part, LD — left dorsal part, I — intrainfundibullary part
6: Arrangement of the kinetosomes that give rise to the short and thick cilia which are pointed by the arrow
7: See text figure
8, 9: Photomicrograph of the right ventral primordium (rvp) in the stomatogenesis of a specimen of *E. caudatum*
10: Detail of the stomatogenesis in a specimen of *E. longinucleatum*. The dorsal extremes of the left dorsal primordium (ldp) and the left ventral primordium (lvp) join together
11: The aparition of the kinetosomes which later give rise to the short cilia can be observed in this photomicrograph
12, 13: Detail of both primordia and the interkinetal fibres
14: Photomicrograph of *E. caudatum* showing the polybrachykinety of the proter and the polybrachykinety of the opisthe. Macronucleus and micronucleus of both ones are also seen



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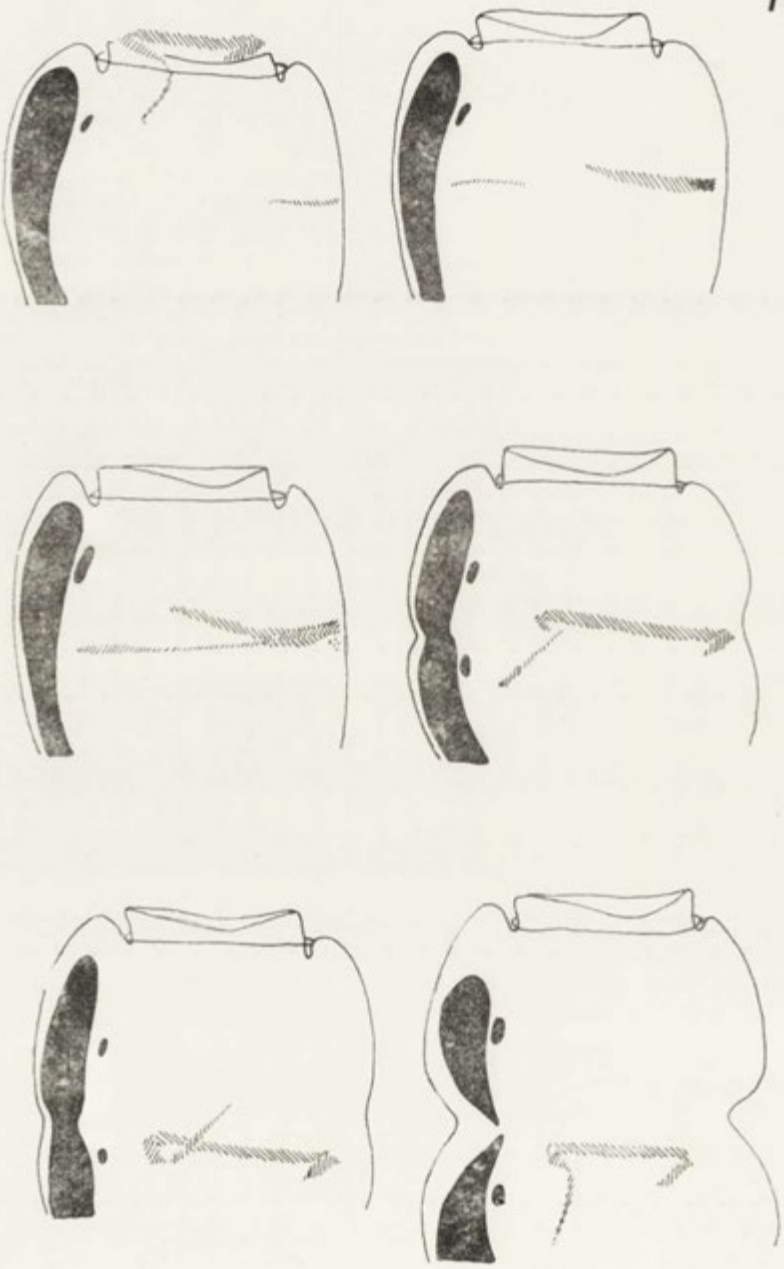
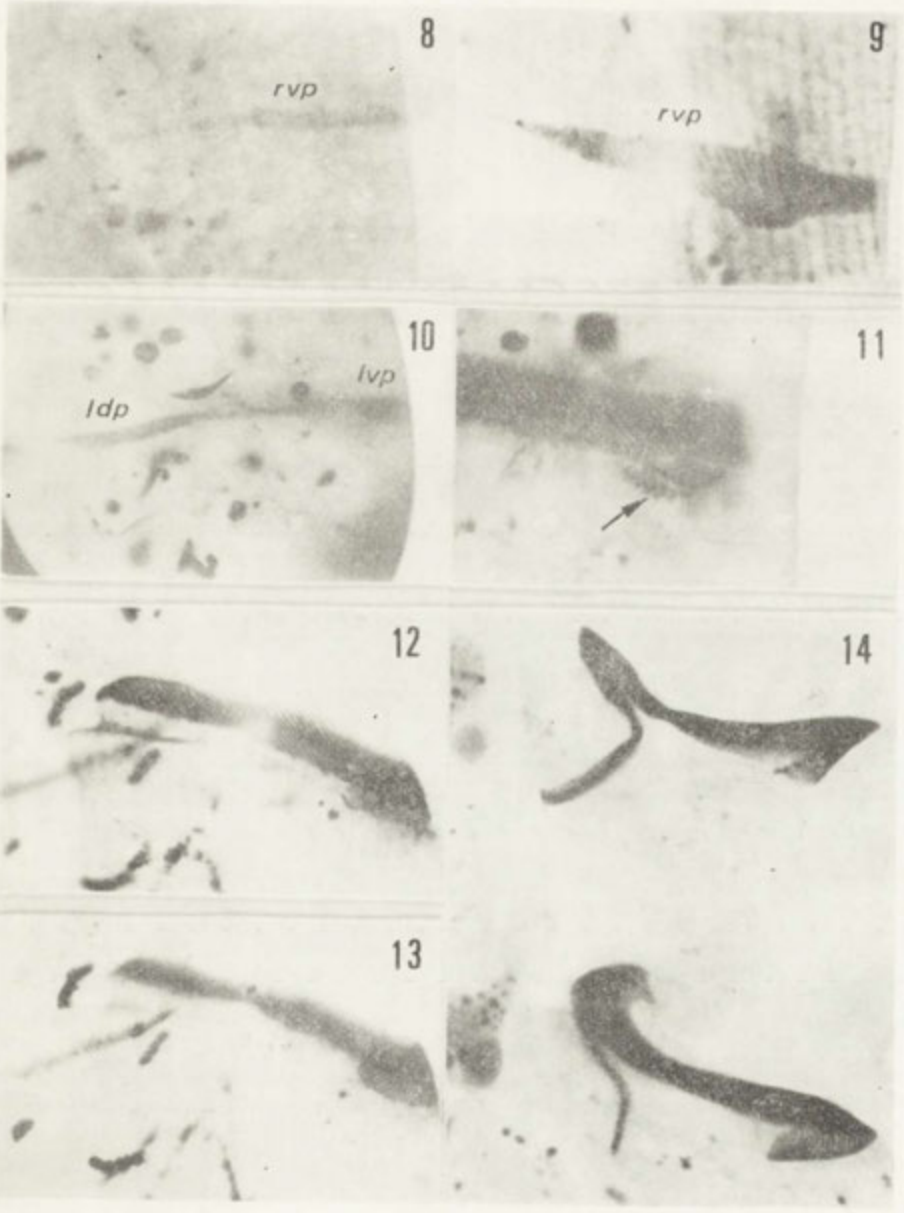


Fig. 7. Semidiagrammatic scheme of the stomatogenesis in the genus *Entodinium*. rvp — right ventral primordium, ldp — left dorsal primordium, lvp — left ventral primordium, pbk — polybrachykinety. Arrow points a group of kinetosomes



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Ultrastructure of the Nuclei of the Lower Ciliate *Trachelocerca variabilis* Kovaleva

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Synopsis. The fine structure of the micronuclei and macronuclei of the multinucleate psammophilic ciliate *Trachelocerca variabilis* (order *Karyorelictida*) was studied. The micronuclei are surrounded by an envelope with a very narrow perinuclear space between the outer and inner membranes. The nuclear envelope is underlain from the inside with an amorphous nuclear lamina. The micronuclei contain densely packed, about 10 nm thick DNP fibrils, which are assembled into thicker strands. Labyrinth-like bodies occur inside widened lacunae between the chromatin strands. Morphologically they resemble the kinetochore material which persists in the interphasic micronuclei of *Trachelocerca geopetiti* and *Tracheloraphis crassus*. Each macronucleus contains a single large chromocenter where all the condensed chromatin of the nucleus is assembled. The structure of the macronuclear chromocenter is similar to that of the micronuclear chromatin, except that no individual strands are visible in it. The macronuclear nucleoli have a typical fibro-granular structure. Each macronucleus contains a nuclear body in form of a large sphere formed by densely packed fibrils, less than 8 nm thick. The sphere is closely adjacent to nucleoli and is often completely covered by a layer of nucleolar granules.

The object of this investigation, the multinucleate ciliate *Trachelocerca variabilis* Kovaleva, 1966, belongs to the family *Trachelocercidae*, order *Karyorelictida* (Corliss 1979). This typical representative of the marine psammophilic fauna (Fig. 1) has first been found at the sandy beaches of the Crimea peninsula, Black sea (Kovaleva 1966), and was later observed also at the Bulgarian coast of the same sea (Kovaleva and Golemansky 1979).

Among the lower ciliates (class *Kinetofragminophora*), the representatives of three families comprising the order *Karyorelictida* (*Trachelo-*

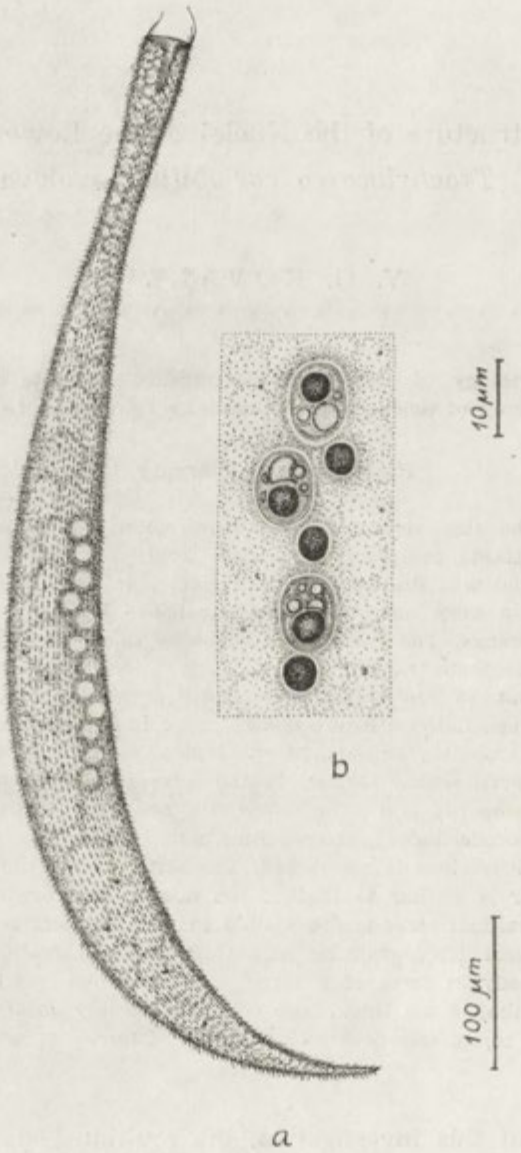

Fig. 1. *Trachelocerca variabilis* Kovaleva: a — general aspect of a living specimen, b — fragment of the nuclear apparatus (total preparation, Feulgen staining)

cercidae, *Loxodidae* and *Geleiidae*) display a primitive organization of their nuclear apparatus. The macronuclei of the karyorelictids are relatively poor in chromatin, and neardiploid according to their DNA content (Kovaleva and Raikov 1978, Bobyleva et al. 1980). These paradiploid macronuclei sharply differ, both morphologically and funct-

ionally, from the massive highly polyploid macronuclei of other *Kinetofragminophora* and of all higher ciliates. The karyorelictid macronuclei belong to the vesicular type of nuclei (Raikov 1967, 1982), their main functional peculiarity is their complete inability to divide. During each cytokinesis, the never-dividing macronuclei are mechanically segregated between the two daughter cells in a more or less equal proportion, while new macronuclei regularly develop from some of the previously divided micronuclei. During this phenomenon, the micronuclei differentiate into macronuclear primordia.

The nuclear apparatus of a number of karyorelictid species has already been studied electron microscopically, including that of several representatives of the family *Trachelocercidae* (for reviews see Raikov 1982, 1985). These investigations have shown that almost all paradiploid macronuclei so far studied contain a definite set of structural components, which includes the nuclear matrix, the nucleoli, the chromatin elements, and the "nuclear bodies". Nevertheless, the karyorelictid macronuclei vary considerably in their fine structural pattern. Different species show a strong diversity as to the number, size, and position of the nucleoli inside the nucleus, the degree of condensation of the chromatin and the position of formed chromatin elements (chromocenters or chromatin bodies) in respect to other nuclear components, and, finally, the type of nuclear bodies (which may be either spheres or crystalloids).

The present paper continues the series of comparative ultrastructural studies of the nuclei of various *Karyorelictida*.

Material and Methods

The material on *T. variabilis* has been collected in June-July 1979 at the Bulgarian coast of the Black sea (namely, at the South side of the Sozopol beach) during an expedition organized by the Institute of Zoology, Bulgarian Academy of Sciences. The ciliates were extracted from samples of the surface layers of sandy sediment, taken at water depths 10-30 cm. They were fixed either with 2% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4), containing 0.003 M calcium chloride and 15% saccharose, or with 4% glutaraldehyde in the same buffer but containing only 8% saccharose. The fixations lasted 20 min at room temperature. Thereafter, the ciliates were pre-embedded into agar gel and conserved in this form in 70% ethanol until the end of the expedition. Then, after dehydration in alcohols of increasing concentration and in acetone, the agar blocks were embedded in a Epon-Araldite mixture. The sections, made on a LKB ultramicrotome with a glass knife, were stained with saturated aqueous uranyl acetate (2-3 h) and lead citrate (15 min) and viewed in a Tesla BS-500 electron microscope.

The technique of regressive staining of the sections with uranyl acetate —

EDTA — lead citrate after Bernhard (1969) was also used. Before staining, the sections were oxidized for 30 min with 0.3% hydrogen peroxide. Then, the sections were stained 1 h with uranyl acetate, treated with EDTA (30-45 min), and counterstained with lead citrate (10-15 min). The solution of 0.2 M EDTA (pH 7.0) was prepared according to Burglen (1974).

Results

Light Microscopy

Trachelocerca variabilis is a large vermiform ciliate which is moderately contractile, when stretched, it is 1000 to 1800 μm long. The mouth is funnel-shaped, terminal, with a longitudinal slit (Fig. 1 a). The nuclear apparatus consists of separate macro- and micronuclei which lie free in the middle part of the ciliate's body, forming a longitudinal row (Fig. 1a). The number of nuclei varies in *T. variabilis* rather widely: From 2 to 14 macronuclei and from 1 to 7 micronuclei.

The micronuclei are spherical, about 5 μm in length, they brilliantly stain with the Feulgen technique (Fig. 1 b).

The macronuclei are oval and measure 8-10 μm in length. Each of them contains a single, large, sharply Feulgen-positive chromocenter, about 4.5 μm in diameter (Fig. 1 b). According to both their size and Feulgen stainability, the chromocenters are very much like micronuclei when observed in light microscopical preparations. The background of the macronuclei is, on the contrary, almost Feulgen-negative, as are the nucleoli and the spherical inclusions into the macronuclei. Practically all the macronuclear DNA thus seems to be condensed and packed into a single large chromocenter.

Fine Structure of the Nuclei

The micronuclei. Plate I 1 presents a survey view of a micronucleus and a nearby macronucleus, and Pl. I 2, a general view of a micronucleus.

The micronuclear envelope is formed by two membranes which are so closely adjacent that they look as a single fused membrane. The perinuclear space between the inner and the outer membranes is discernible only around the rather sparse pores (Pl. I 2, 3, at arrows). From the nuclear side, the micronuclear envelope is uniformly underlain with an amorphous layer (nuclear lamina), which is about 85 nm thick and separates the inner membrane from the chromatin (Pl. I 2, 3).

The micronuclear chromatin looks in the sections as a cut threadball of tightly interlaced, rather thick (about 120 nm) strands of high electron density (Pl. I 2). At higher magnifications, individual densely packed DNP-fibrils which are about 10 nm thick can be discerned in the strands. Inside the "threadball", the strands are separated by either narrow clefts or rather large cavities filled with clear nuclear matrix. Many of these cavities contain accumulations of material which can be termed "labyrinth-like bodies" (Pl. I 1-3, II 4). These are clearly different from the strands of compact chromatin, filling the micronucleus, in both their structure and electron density. The labyrinth-like bodies consist of two components: the denser, which forms profiles of narrow curved lamellae about 20-30 nm thick, and the looser, which corresponds to fine fibrils situated between the lamellae (Pl. I 3, white arrow). No special envelopes exist around the labyrinth-like bodies. Up to 12 such bodies can be counted on a single section through a micronucleus.

The macronuclei. Like the optical microscope, the electron microscope clearly reveals a single large chromocenter inside each macronucleus, this may occupy almost a half of the nuclear volume (Pl. II 4, III 7). The chromocenter is sharply delimited from the karyolymph and usually has a more or less jagged or lobulated surface (Pl. II 4, III 7, 8, IV 12). It consists of densely packed DNP fibrils of a high electron density (Pl. II 6, IV 12). Unlike the micronuclear chromatin, the macronuclear chromocenter looks like a compact or spongy mass in which no individual strands can be discerned, only narrow clefts and some vacuoles are seen on sections inside it (Pl. II 4, III 7, IV 12). The vacuoles may have different contents: some of them are filled with loose, coarsely fibrous material which structurally resembles the nuclear matrix (Pl. III 7, IV 12, single arrow), while others contain thinner and more densely packed filaments (Pl. III 7, IV 12, double arrow). Finally, still other vacuoles display homogeneous contents (Pl. III 7, IV 12, broken-like arrow).

Neither small chromatin bodies nor aggregates of chromatin fibrils outside the chromocenter could be revealed. It is likely that all the condensed chromatin of a macronucleus of *T. variabilis* is united into a single compact chromocenter.

Many chromocenters display a rather large inner cavity with very sinuous outlines, which is filled with the karyolymph. Some micrographs demonstrate that this cavity is actually continuous with the karyolymph outside the chromocenter (Pl. III 8).

Along with the chromocenter, each macronucleus contains nucleoli which may number up to 10 (usually 5-6) per nucleus. The nucleoli are as a rule spherical or slightly polymorphous and measure from 0.6 to

1.6 μm in diameter (Pl. II 4, 5). They have a typical fibro-granular structure but lack apparent nucleolar organizer regions. Many nucleoli are closely adjacent to the nuclear body, i.e., the sphere (Pl. I 1, II 6, III 7). Along with such typical nucleoli, the macronuclei may contain also micronucleoli (Pl. II 5, at arrows). These measure only about 0.1 μm and consist of only the granular material. Finally, very small accumulations of granules (30-50 nm in size) as well as single granules of RNP (8-10 nm in diameter) also occur, these are usually localized at the periphery of the spherical nuclear body (Pl. II 5, 6, III 7, IV 11, 13, 14). Following the regressive staining of sections of the macronucleus (according to Bernhard), the nucleoli which contact the nuclear body retain their contrast while the chromocenter becomes rather strongly bleached (Pl. III 9). This confirms the RNP nature of the nucleolar components.

The spherical nuclear body ("sphere") is, along with the chromocenter, a characteristic component of the macronuclei of *T. variabilis*, it was always single and present in all macronuclei studied. The sphere usually lies near the other end of the nucleus in respect to the chromocenter and occupies almost one third of the macronuclear volume. The diameter of the spheres met with in electron micrographs varied from 3 to 4 μm. The sphere differs from both nucleoli and chromocenters by its smooth outlines and rather low electron density (Pl. I 1, III 7, IV 11). The sphere has no special envelopes and lies directly in the nuclear matrix. In some cases, the sphere is almost completely bordered with RNP granules of about 12 nm in diameter, which lie in two or three layers directly on its surface (Pl. IV 11, 13). At low magnifications, the sphere appears to be quite structureless, however, with relatively high resolutions it can be seen to consist of very thin densely packed filaments (less than 8 nm in diameter), which have low electron density (Pl. IV 11, 13). On one side, the sphere always borders upon the chromocenter (Pl. III 7, 8), but this seems to be merely a fortuitous contact: the micrographs never show any specific association between the sphere and the DNP fibrils. In all the cases examined, such close associations exist, however, between the sphere and the nucleoli which are usually flattened against the sphere. The sphere is associated either with the entire granular surface of a nucleolus (Pl. II 6, III 7) or with isolated RNP granules (Pl. IV 11, 13) which seem to spread from the nucleoli onto the sphere surface (Pl. IV 14).

The macronuclear envelope has in *T. variabilis* a typical structure, it carries pores which show central granules in grazing sections (Pl. III 10). The pore diameter is 60 nm. Besides its own envelope, each macronucleus is surrounded by an additional cytoplasmic membrane.

Discussion

According to their fine structure, the micronuclei of *T. variabilis* belong to "spermal" type nuclei, the same as the micronuclei of all other karyorelictids so far studied (Raikov 1978, 1982). These nuclei typically show a dense packing of the condensed chromatin in a small volume. The absence of nucleoli from the micronuclei of the karyorelictids (including *T. variabilis*) indicates a transcriptional inertness of these nuclei.

The 0.12 μm thick dense strands filling the micronuclei of *T. variabilis* seem to be chromosomes which are drawn together but show almost no interphasic decondensation. Their chromatin is formed by tightly packed, some 10 nm thick fibrils (which are likely to be nucleosomal fibrils). Such a structure of the chromatin is typical of the micronuclei of all karyorelictids investigated (Raikov 1985), except those of *Geleia orbis* in which the chromatin consists of granules of a supranucleosome (=nucleomere) size, i.e., about 30 nm (Raikov 1984).

Of special interest are the labyrinth-like bodies discovered in the micronuclei of *T. variabilis*. They look somewhat like the lamellar structures found inside the lacunae of the chromatin mass in the micronuclei of *Trachelocerca geopetiti* (Raikov and Kovaleva 1981), and the ring-shaped lamellae occurring in the micronuclei of *Tracheloraphis crassus* (Raikov and Karadzhan 1985). A detailed ultrastructural and ultracytochemical investigation of the lamellar structures in the interphasic micronuclei of *T. geopetiti* and their comparison with the kinetochores of mitotic chromosomes of the same species enabled the authors to consider these structures as chromosomal kinetochores which persist during interphase. It is unfortunately not possible to solve now the question about the nature of the labyrinth-like bodies in the micronuclei of *T. variabilis*, since only morphological data and only data on interphasic micronuclei are presently available. However, taking into account their similarity with persisting kinetochores in other species, the homology of these structures cannot be excluded.

It may be supposed that conservation of clearly visible kinetochores in the interphasic nuclei is a phenomenon characteristic of karyorelictids possessing large chromosomes which are poorly decondensed during the interphase. In our case, the micronuclei of the three species involved (*T. variabilis*, *T. geopetiti*, *T. crassus*) are rather similar as to the packing of the chromatin inside the nucleus. They differ only in the presence of an amorphous nuclear lamina beneath the micronuclear envelope, which exists in *T. variabilis* but not in the other two species. Therefore, the supposition that the labyrinth-like bodies of the micronuclei

of *T. variabilis* are accumulations of kinetochore material persisting in interphasic nuclei appears likely.

Among the other protozoans, the presence of kinetochores in interphasic nuclei has been observed in certain *Hypermastigida* (Hollande et Carruette-Valentin 1971, Kubai 1973).

The macronuclei of *T. variabilis* clearly belong to vesicular nuclei, according to Raikov's (1982) classification, in this respect they do not differ from the somatic nuclei of other karyorelictids containing a paradiploid quantity of DNA.

The existence of a single large compact chromocenter which apparently encloses, in condensed state, almost all the chromatin of the nucleus, is a rather peculiar feature of the macronuclear structure in *T. variabilis*. Macronuclei of similar organization (with a single large chromocenter) occur also in the lower ciliate *Kentrophoros fistulosum*, family *Loxodidae* (Raikov 1972). Nuclei of such a type may be called karyosomal (Raikov 1982). Being poorly differentiated in the somatic direction, they seem to stand, according to their functional activity, nearer to the micronuclei, i.e., to transcriptionally inert nuclei, than macronuclei of other karyorelictids so far investigated. In most karyorelictids, only a part of the chromatin is normally condensed and packed into chromocenters, which vary in number, size, and structure from one species to another (reviewed by Raikov 1985). The other part of the chromatin is always decondensed. The relation between these two parts generally reflects the degree of the transcriptional activity of the chromatin. The macronuclear chromatin of *Geleia orbis* (Raikov 1984) seems to have the highest transcriptional activity, since it is completely decondensed.

It is known that the degree of nuclear activity influences not only the state of the chromatin but also the development of the nucleolar apparatus (Chentsov and Polyakov 1974). The least active macronuclei of *K. fistulosum* contain only few small nucleoli which have purely fibrillar structure (Raikov 1972). On the other hand, the highly active macronuclei of *G. orbis*, possessing a fully decondensed chromatin, display a large number (more than 100) of nucleoli with definite fibrillar cores and granular cortexes (Raikov 1984). The macronuclei of *T. variabilis* occupy an intermediate position as to the degree of development of the nucleolar apparatus. Their nucleoli are typically fibro-granular (unlike those of *K. fistulosum*), and both the number and the size of the nucleoli are near the average ones.

The macronuclei of the karyorelictids consistently display nuclear bodies which may take the form of either spheres or crystalloids (Raikov 1985). In the macronuclei of *T. variabilis*, the nuclear body is

a sphere. The sphere of this species most closely resembles in its fine structure the spheres of *Trachelonema sulcata* (Kovaleva and Raikov 1970, 1973), *Tracheloraphis dogieli* (Raikov 1974), *Trachelocerca geopetiti* (Raikov and Kovaleva 1981), *Tracheloraphis crassus* (Raikov and Karadzhan 1985), and *Geleia orbis* (Raikov 1984). In these species, the macronuclear spheres are formed by densely packed fibrils, though of varying thickness: either 7-8 nm (*T. sulcata, T. dogieli, T. geopetiti*, and *T. variabilis*), or 10-12 nm (*T. crassus, G. orbis*). Moreover, in some species the spheres contact the condensed chromatin (*T. sulcata, T. dogieli*), while in other species they are associated with nucleoli (*T. geopetiti, T. crassus, G. orbis*). The sphere of *T. variabilis* belongs to the latter group. Ultracytochemically, the proteinaceous nature of the spheres has been demonstrated in *T. dogieli* and *T. geopetiti* by their complete digestion with pronase (Raikov and Kovaleva 1980, 1981), while in *G. orbis* and *T. crassus* the sphere seems to consist of ribonucleoprotein (Raikov 1984, Raikov and Karadzhan 1985).

In the lack of cytochemical data, we cannot judge about the chemical nature of the sphere in *T. variabilis*. Yet, is seems likely that nucleolar RNP may take part in its formation, since the sphere of our species regularly contacts the surface of one or several nucleoli and is surrounded by loose RNP granules which form a layer on its very surface.

Unfortunately, the functional significance of the macronuclear spheres remains so far unknown. There is only a supposition (Raikov 1985) that the spheres may represent stocks of proteins, in some cases of chromatin-associated proteins, in other cases, of nucleolar proteins and/or RNPs.

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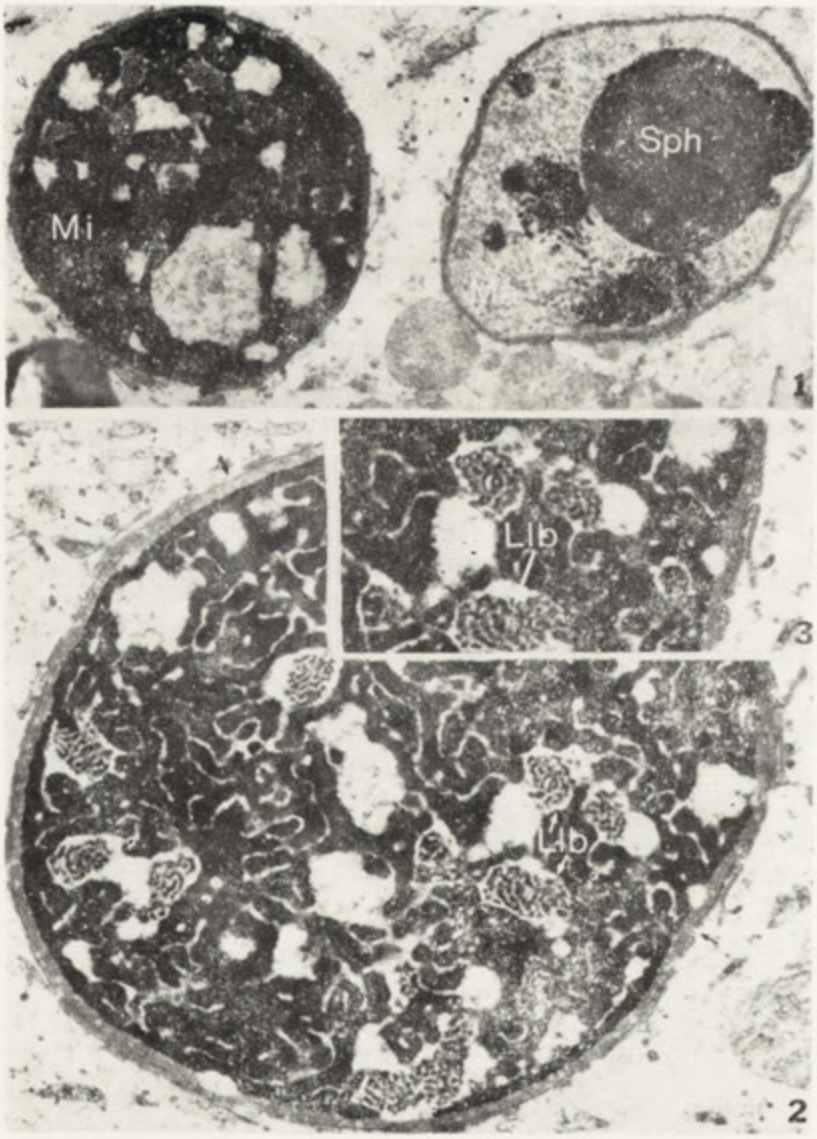
EXPLANATION OF PLATES I-IV

Abbreviations used in all plates:
Ma — macronucleus, Mi — micronucleus, N — nucleolus, F — fibrillar zone, G — granular zone of a nucleolus, Chr — chromocenter, Sph — spherical nuclear body ("sphere"), Llb — labyrinth-like body in a micronucleus

1: Survey micrograph of a micronucleus and a macronucleus. The Mi shows labyrinth-like bodies, the Ma, a large sphere. 11 000 ×
2: Micronucleus with labyrinth-like bodies. Pores of the nuclear envelope at arrows. 21 600 ×
3: Part of the same micronucleus at higher magnification. The white arrow shows the loose fibrillar component of the labyrinth-like bodies. 27 000 ×
4: Part of a macronucleus showing the large chromocenter and several nucleoli, and part of a micronucleus with labyrinth-like bodies. 8000 ×
5: Three nucleoli displaying fibro-granular structure, and two micronucleoli (at arrows) formed by only the granular component. 43 200 ×
6: Contact between the sphere and the granular cortex of a nucleolus. 48 000 ×
7: Macronucleus showing the large chromocenter, the sphere, and three nucleoli. 18 000 ×
8: Chromocenter with a cavity filled with karyolymph. 27 000 ×
9: Fragment of the sphere and the chromocenter, a nucleolus contacting the sphere. Bernhard's technique. 27 000 ×
10: Tangential section of the macronuclear envelope, showing pore complexes with central granules. 32 000 ×
11: Part of a sphere coated with nucleolar granules. 48 000 ×
12: Part of a chromocenter showing three kinds of vacuoles; with coarse fibrils (simple arrow), with fine fibrils (double arrow), and with homogeneous contents (broken-line arrow). 29 700 ×
13: Margin of a sphere showing its finely fibrillar structure and a coat of nucleolar granules on its surface. 48 000 ×
14: Close contact between a sphere and the nucleolar granules: the latter seem to spread on the sphere surface (tangential section). 27 000 ×



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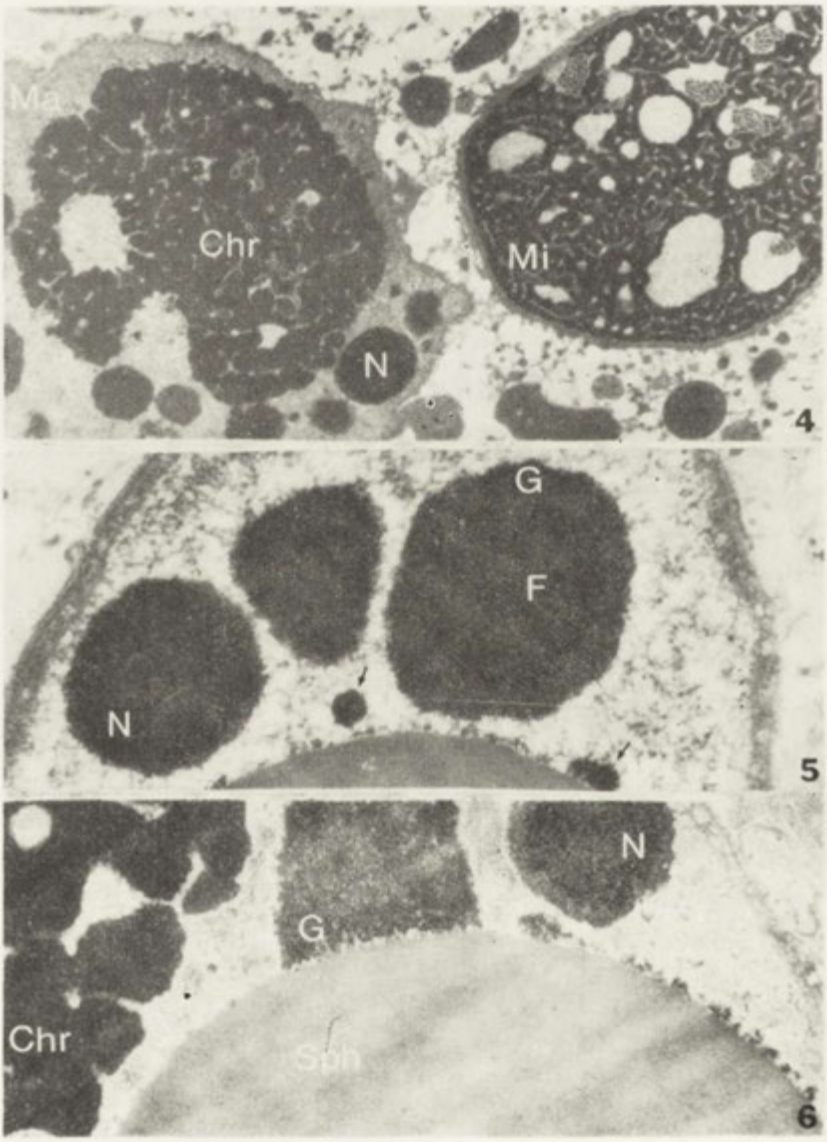

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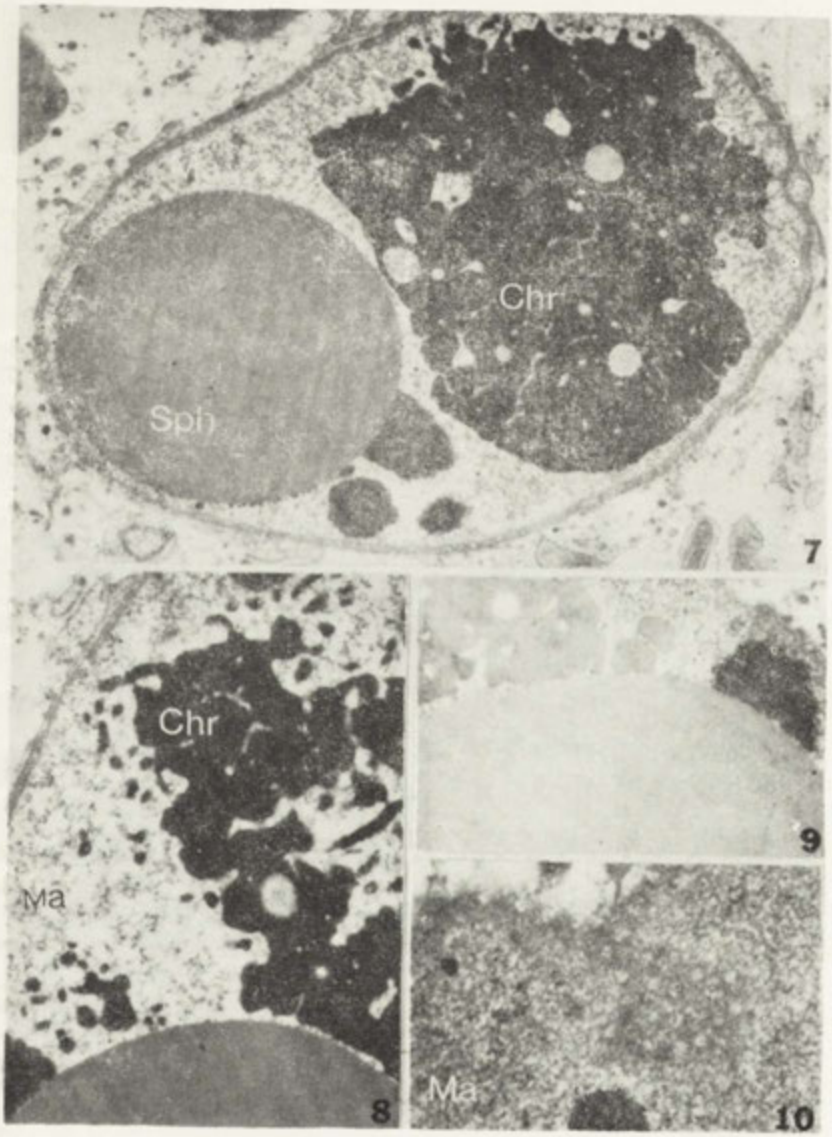

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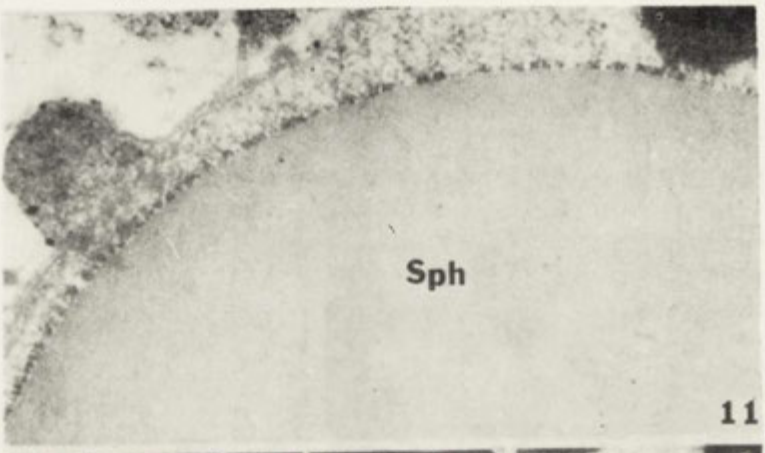

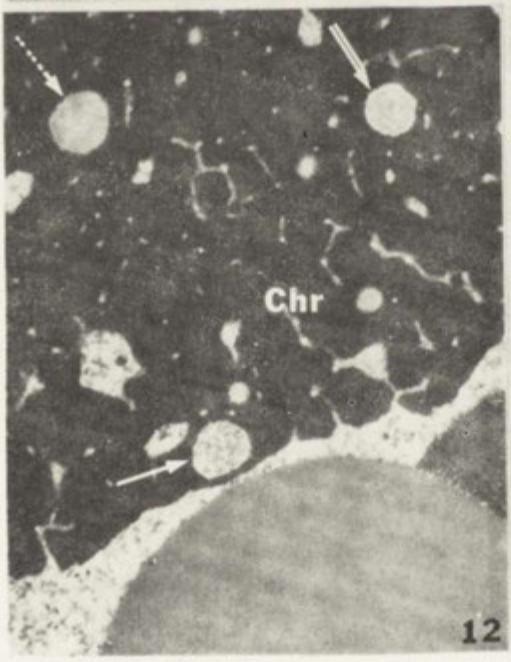

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Development of the Macronucleus Following Conjugation of the Ciliate *Dileptus anser*. I. Cytophotometric Study of the Changes in DNA Content of the Macronuclear Anlagen

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Synopsis. The changes in DNA content during the development of the macronucleus following conjugation in *Dileptus anser* have been investigated by cytophotometry of Feulgen stained isolated nuclei. Each of the earliest macronuclear anlagen measured contained about 14 arbitrary units (a. u.) of DNA, whereas a diploid micronucleus contained 10-13.5 a. u. Four stages can be distinguished in the course of macronuclear development. During the I stage, the mean total amount of DNA in the four macronuclear anlagen, typical of the early exconjugants of *D. anser*, increases from 58 a. u. to 630 a. u., indicating that more than three rounds of DNA replication occur. During the II stage, the DNA synthesis in the anlagen continues, but the anlagen start dividing. By the end of this stage, most exconjugants contain 16 anlagen, and the mean total DNA quantity in them reaches 1700 a. u. At the III stage, the exconjugants contain 32-40 macronuclear anlagen, their total DNA content is, however, 1.3-1.4 times less than that at the stage of 16 anlagen. The decrease of the DNA amount at the III stage may mean genetic diminution leading to a qualitative modification of the gene complement of the adult macronuclear apparatus of *D. anser*. During the IV stage of macronuclear development, a linear increase of the total DNA content of the macronuclear anlagen occurs again. Before the first post-conjugation cell division the total amount of DNA in the new macronuclear apparatus is about 90 times that of the early exconjugants. This requires about 7 rounds of DNA replication, of which 5 rounds occur before and 2, after the elimination of a part of DNA of the anlagen.

The somatic nucleus (macronucleus) of the ciliates is a convenient model for studying the transformations of the genetic apparatus of a cell nucleus coupled during its somatic differentiation. Especially important

for the comprehension of the structure of the macronucleus is the process of differentiation of the macronuclear primordia ("anlagen"), which occurs during conjugation of the ciliates. This phenomenon involves the replacement of the old macronucleus by a new one, which is formed from certain products of division of the diploid synkaryon.

The developing macronuclear anlage undergoes quite a series of transformations before it becomes a mature macronucleus, usually very rich in DNA. Several authors have clearly demonstrated that deep changes in the genetic apparatus of the developing macronucleus occur in the course of this process. Moreover, these changes seem to be different in various ciliate taxa, possibly at various steps of evolution of the ciliates. In some ciliates, the chromosomes of the anlage undergo fragmentation followed by drastic genetic diminution of many loci and differential replication of other loci, in other ciliates, the macronucleus is likely to conserve a manifold repeated but unreduced or weakly reduced genome (Ammermann 1973, Murti 1976, Prescott et al. 1979, Gorovsky 1980, Raikov 1982, Yao 1982). Comparative investigations of the structure and development of the macronucleus in primitive taxa of the ciliates are therefore essential for understanding the ways of evolution of the somatic nuclei.

This paper presents the results of a cytophotometric study of the DNA content during the development of the macronucleus following conjugation in a multinucleate ciliate *Dileptus anser*, which belongs to the subclass *Gymnostomata*, a pivotal group in the phylogenetic system of the ciliates (Corliss 1979). The morphology of the nuclei of *D. anser* and the reorganization of its nuclear apparatus during conjugation have been described by Vinnikova (1974).

Material and Methods

Dileptus anser belongs to the lower ciliate order *Haptorida*, family *Trachelliidae* (Corliss 1979). The nuclear apparatus of this species is characterized by a fragmented macronucleus, consisting of 200 to 600 macronuclear fragments and 6 to 12 micronuclei (Hayes 1938).

The work has been done using exconjugant cells of *D. anser*, which were formed after conjugation between complementary mating type clones 4d and 12 of the culture collection of the Laboratory of Cytology of Unicellular Organisms of the Institute of Cytology. The ciliates were grown according to the method described by Nikolayeva (1968).

The DNA content of the macronuclear anlagen was measured in isolated nuclei of the exconjugants at various stages of anlagen development, from the moment of separation of the conjugants up to the first post-conjugation cell division. To isolate the nuclei, exconjugants were individually lysed on a slide

in small drops of the following mixture: aqueous solution (1 : 200) of Triton X-100, 7 ml, spermidine solution (0.5 mg/ml), 3 ml, 1 M saccharose, 0.5 ml. The isolated nuclei were fixed with vapours of 40% formalin during 10 min, air-dried, and Feulgen stained according to the standard technique (hydrolysis in 1 N HCl for 6 min at 60°C).

The cytophotometric measurements of the DNA content of the nuclei were carried out using a two-wave cytophotometer (Rosanov and Selivanova 1968) operated in the single-wave regime at the margin of the absorption band, wavelength 505 nm (Selivanova and Archipov 1974). The quantity of the DNA-fuchsine compound was calculated in arbitrary units, as the product of the measured optical density of the given nucleus by the diameter of the probe. The diameter of the probe was selected in order that the nucleus to be measured completely fits into the circumference of the probe. The macronuclear anlagen of 20-28 specimens were measured for each exconjugants age.

Results

The vegetative specimens of the clones studied (4d and 12), which are complementary by their mating types, contain 250-300 macronuclei. Most of the cells enter conjugation approximately 2-2.5 h after the clones have been mixed (at 22°C). The conjugants separate rather synchronously 20-22 h after the formation of the pairs. At 22°C and with sufficient food supply, the development of the exconjugants (from the moment of separation, hereafter called zero hour, to the first division of the exconjugant cell) lasts for about 54-56 h. By the moment of separation of the partners each nascent exconjugant cell contains, as a rule, four early macronuclear anlagen. The normal number of macronuclei, typical of vegetative specimens of *D. anser*, is attained in result of several successive divisions of all anlagen available in the cell and without division of the exconjugant itself. The macronuclear apparatus of *D. anser* thus originates from several macronuclear anlagen, not from a single one. By the first post-conjugant cell division, the late exconjugants of *D. anser* contain about 250 new macronuclei.

The results of cytophotometric measurements of the DNA content at different stages of macronuclear development, beginning with early exconjugants up to the first post-conjugation cell division, are presented in the histograms (Figs. 1 and 2) and summarized in the diagram (Fig. 3). Four stages can be distinguished in the process of macronuclear development of *D. anser*.

Stage I of anlagen development starts with separation of the conjugants and last for about 18-20 h. The four macronuclear anlagen, found in most newly separated exconjugant cells, have spherical shape and a diameter of 2-3 μm, they faintly and homogeneously stain with

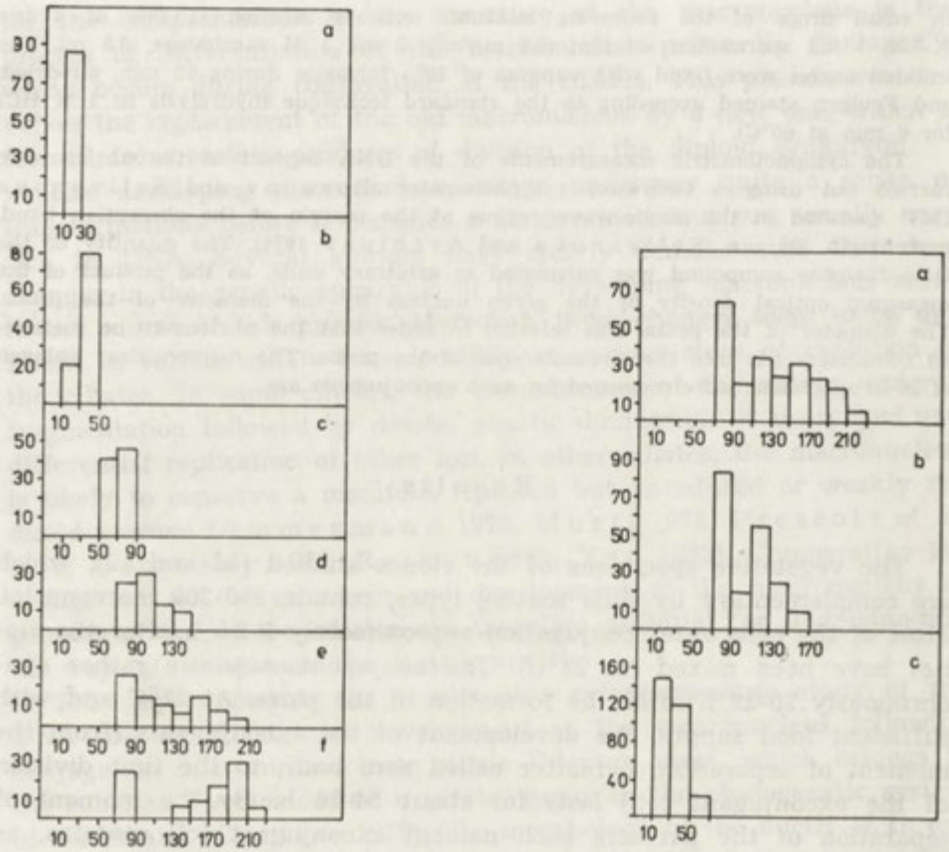

Fig. 1. DNA content of individual macronuclear anlagen of *Dileptus anser* at the stage of four anlagen: *a* — immediately after the separation of the conjugants (0 hours), *b* — 3 h, *c* — 7 h, *d* — 11 h, *e* — 15 h, *f* — 18 h after the separation. *Abscissa:* DNA content in arbitrary units, *ordinates:* number of anlagen measured

Fig. 2. DNA content of individual macronuclear anlagen at the stage of eight anlagen (a), 16 anlagen (b), and 32-40 anlagen (c). *Abscissa:* DNA content (arbitr. units), *ordinates:* number of anlagen measured

the Feulgen method. At this time, the total amount of DNA in the four anlagen together is about 58 a.u. The difference between individual anlagen as to their DNA content is rather small at this moment, especially between the anlagen of a single exconjugant. Also in different newly separated exconjugants, individual anlagen usually contain between 14-15 a.u. of DNA. Thus, immediately after separation of the conjugants, all macronuclear anlagen are practically at the same phase of development.

At later times of development, the extent of variation of individual

anlagen according to their DNA content strongly increases when the whole population of exconjugants is considered (Fig. 3), but remains rather narrow within each given exconjugant cell throughout stage I. Since the four anlagen of each cell continue to contain approximately equal amounts of DNA, the overall variability of the DNA content in the anlagen evidently increases mainly due to the growing asynchrony of development between individual exconjugants.

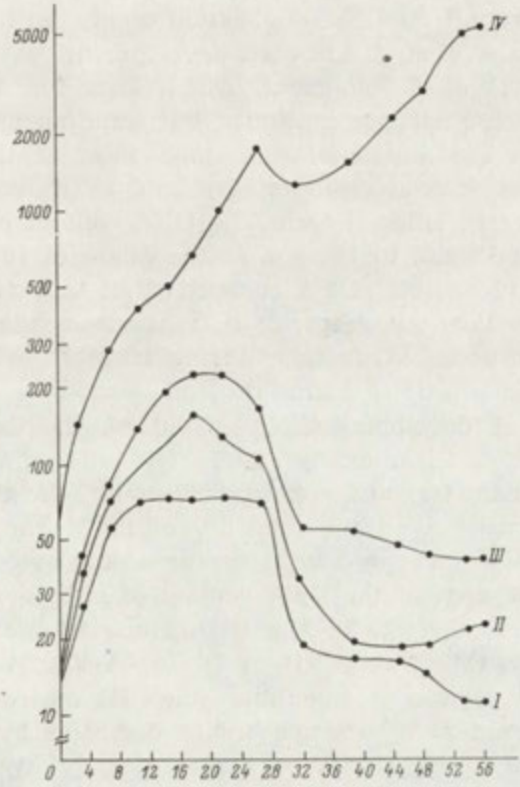

Fig. 3. Minimum (I), maximum (III) and mean DNA content (II) in individual macronuclear anlagen from the moment of separation of the conjugants up to the first post-conjugation cell division. IV — Total mean DNA content in all anlagen of a exconjugant at the same time. *Abscissa*: time in hours, *ordinate*: DNA content in arbitry units (logarithmic scale)

The DNA content of the micronuclei measured in early exconjugants varied between 10 and 13.5 a.u. If the latter quantity corresponds to the post-synthetic DNA level of the diploid micronucleus, the earliest macronuclear anlagen investigated in this study would contain only about 4 c of DNA, i.e., they would have undergone only one round of DNA replication before the conjugants separated.

At later phases of stage I of development, the four macronuclear anlagen grow and gradually accumulate DNA (Fig. 1, 3). By the end of stage I, the anlagen deeply stain with Feulgen and reach a diameter of about 10 μm. Some individual anlagen accumulate by the end of stage I (18 h) up to 200 a.u. of DNA, while the mean DNA content of a single anlage reaches 157 a.u. (Figs. 1, 3). The mean total amount of DNA (in the four anlagen of an exconjugant together) increases during stage I from 58 a.u. to 630 a.u. in 18 h old exconjugants. This means that more than three rounds of DNA duplication occurs in the macronuclear anlagen in the course of stage I of their development.

During stage II of development, which lasts for about 10 h, the DNA synthesis in the anlagen continues, but simultaneously the anlagen start dividing. At the middle of this stage, most of the exconjugants already have eight macronuclear anlagen, and by its end, the exconjugants usually have 16 anlagen each. The DNA content of individual anlagen varies from 72 a.u. to 167 a.u. at the phase of 16 nuclei (age 27-30 h), while the mean total DNA content of all the anlagen of an exconjugant reaches 1700 a.u. (Figs. 2, 3). This means that less than two DNA duplications occur at average during stage II, while all anlagen are likely to divide two times during the same period.

At stage III of development (age 32-40 h), the exconjugant cells contain 32-40 macronuclear anlagen each. The anlagen are rather small (about 4 μm in diameter) and spherical. Visually, the density of packing of their chromatin and the intensity of its staining with Feulgen appears decreased as compared with the preceding stage. Cytophotometrically, an abrupt drop of the DNA content of individual macronuclear anlagen is recorded (Figs. 2, 3). The DNA amounts in single nuclei are strongly variable at this stage (from 16 to 54 a.u., the latter figure being the highest registered one for a stage III macronuclear anlage). The mean DNA content of a single anlage decreases by the 32nd hour to 34 a.u. This decrease cannot be explained only by the division of the anlagen in the absence of DNA synthesis, since at the preceding stage (16 anlagen) they contained at average 100 a.u. of DNA each. The mean total DNA content of all the anlagen of an exconjugant, taken together, also decreases in stage III exconjugants by a factor of 1.3-1.4 (by the 32nd hour) as compared with the stage of 16 anlagen (age 27 h): it drops from 1700 a.u. to 1200 a.u. (Fig. 3). This decrease of the DNA content at the stage of 32-40 macronuclear anlagen is significant at the confidence level of 95%. Consequently, the data on DNA content of the macronuclear anlagen of stage III exconjugants demonstrate that elimination of about 25% of all DNA, which was previously accumulated in the anlagen, occurs at a definite moment of macronuclear develop-

ment in *D. anser*. It is likely that such an elimination may occur during division of the anlagen.

During stage IV of macronuclear development, which lasts for about 15 h (age 40-55 h), the macronuclear anlagen continue to divide. A linear increase of the DNA content of the developing macronuclear apparatus is resumed at this period (Fig. 3). Just before the first post-conjugation cell division (age 54-56 h), the exconjugant cell of *D. anser* contains about 250 spherical new macronuclei. The DNA content of individual macronuclear anlagen remains more or less constant throughout stage IV: it varies from 14 a.u. to 42 a.u. and equals at average about 22 a.u. between hours 39 and 54 of development (Fig. 3). This means that the synthesis of DNA in stage IV macronuclear anlagen almost exactly keeps in pace with their successive divisions, and the two processes thus compensate each other.

The mean total DNA content of the new macronuclear apparatus increases during stage IV from 1200 a.u. to 5200 a.u. (Fig. 3). It consequently reaches a value 90 times higher than that at the moment of separation of the conjugants, which requires, with account of the transitory phase of DNA elimination, seven rounds of DNA replication. Of these, five occur before and two, after the drop in DNA content. Taking into account that the earliest individual anlagen measured were likely to contain already about 4 c of DNA, the overall number of DNA duplications may, however, be eight. The total DNA content of the macronuclear apparatus by the end of development (5200 a.u.) may equal about 1500 c (1 c $\approx$ 3.5 a.u.), and that of its part developed from a single, initially diploid macronuclear anlage, about 375 c.

Discussion

The temporary decrease of the DNA content of the macronuclear anlagen, which occurs at a rather late stage of their development, seems to be the most interesting phenomenon in the course of macronuclear development in *D. anser*. The decrease of the amount of DNA at the stage of 32-40 anlagen as compared with the stage of 16 anlagen is clearly expressed both in the individual anlagen and in the sum of the anlagen present in the cell. In the former, the drop is at average to one-third, despite the fact that the anlagen divide in the meanwhile only once. In the latter, the drop of the total DNA content is at average to 70% of the initial amount (see Fig. 3). This decrease of the DNA content apparently means that elimination of some part(s) of the initial genome occurs during the development of the macronucleus in *D. anser*.

In other words, the genetic apparatus of the future somatic nuclei undergoes a kind of processing.

Diminution of the chromatin in the course of macronuclear development has already been discovered in many ciliates of various taxonomic positions. The most extensive elimination of DNA occurs from the developing macronuclei in the exconjugants of higher ciliates of the order *Hypotrichida*. In *Stylonychia*, *Oxytricha*, *Euplotes* and some other hypotrichs, the macronuclear development begins with polytenization of the chromosomes of the anlage. During this stage, corresponding to the first phase of DNA synthesis, elimination of a large part of the original micronuclear genome already occurs in *Stylonychia*, since only one-third of the chromosomes participate in the further development, and the rest remain condensed and do not undergo polytenization (Ammermann et al. 1974, review: Raikov 1982, and others). Many discrete heterochromatin blocks have been discovered on the polytene chromosomes of *Stylonychia*, these are apparently formed in result of over-replication of some definite DNA sequences. Consequently, disproportional replication of certain parts of the genome of the micronucleus occurs at least twice even at the early stages of development of the macronuclear anlage of *Stylonychia*, namely in form of polytenization of only some chromosomes of the synkaryon derivative and in form of over-replication of some loci in the polytene chromosomes.

The maximum development of the polytene chromosomes in *Hypotrichida* is followed by their transverse fragmentation, which is accompanied by an abrupt decrease of the DNA content, some 93% of the DNA is eliminated from the macronuclear anlage of *Stylonychia*, and the anlage enters the "achromatic" stage of development. It has been demonstrated that the reduction of the DNA content results from breakdown of the DNA (Ammermann 1968) and that elimination of some DNA from all bands of the former polytene chromosomes occurs (Prescott and Murti 1974). The adult macronucleus of these ciliates is formed by additional manifold replication of the remaining parts of the genome which are represented by free gene-sized DNA molecules (second phase of DNA synthesis).

As to the nature of the DNA which is eliminated from the hypotrichid macronuclear anlagen, two points of view presently exist. Prescott and co-authors (Prescott et al. 1971, Prescott and Bostock 1973, Lauth et al. 1976, Swanton et al. 1980, and others) suppose that it is mainly the non-transcribed spacer DNA which is selectively eliminated, the adult macronucleus being formed by manifold amplification of only the free structural genes. According to the other viewpoint, a true gene diminution occurs as well during macronu-

clear development of *Stylonychia* and other hypotrichs (Ammermann et al. 1974).

Elimination of a significant part of the DNA occurs also during development of the new macronucleus of *Chilodonella cucullulus* (Radzikowski 1973). Some 35% of DNA is eliminated from the macronuclear anlage of this ciliate at a late stage of its development. It seems that there is no direct relationship between the systematic position of the ciliate and the mode of development of its macronucleus. Thus, in another species of the same genus, *Ch. uncinata*, both cytophotometric and autoradiographic data indicate a progressive accumulation of DNA throughout the development of the new macronucleus following conjugation (Pyne et al. 1974, Pyne and Gache 1979). A steady accumulation of DNA during macronuclear development has been recorded cytofluorimetrically also in. *Tetrahymena thermophila* (Doerder and de Bault 1975). However, since biochemical differences between the macronuclear and micronuclear DNAs of *Tetrahymena* do exist, one has to admit that selective elimination of certain minor fractions of the micronuclear DNA (in a quantity not exceeding 10% of the original DNA content) must nevertheless occur during the macronuclear development of this ciliate (Gorovsky 1973, 1980, Yokoyama and Yao 1980).

Chromatin diminution during macronuclear development has been discovered also in *Paramecium bursaria* (Schwartz and Meister 1975). As in *D. anser*, two phases of DNA synthesis exist in *P. bursaria*, they are separated by a phase when a loss of a part of the DNA occurs (the "achromatic" stage). However, unlike *D. anser* where DNA diminution occurs at a rather late stage of macronuclear development, the "achromatic" stage in *P. bursaria* is placed early in the anlagen development, when the DNA content of an anlage reaches only six times that of a micronucleus. During chromatin diminution, almost a half of this DNA is lost. Simultaneously, the chromosomes of the macronuclear anlagen of *P. bursaria* become significantly shorter since their arms are deleted and resorbed, so that only the short near-centromeric regions of the chromosomes are further amplified and incorporated into the new macronucleus (Schwartz 1978). In this case also, the chromatin diminution may be true genetic diminution.

A loss of a certain part of the genome of a micronucleus at the beginning of its transformation into a macronuclear anlage, followed by partial replication of some of the conserved DNA fractions during the further development and aging of the macronucleus, have been demonstrated by cytophotometry also in a lower ciliate of the order *Karyorelectida* — *Trachelonema sulcata* (Kovaleva and Raikov 1978).

Thus, most of the ciliates studied display an elimination of a smaller or larger portion of the initial DNA during macronuclear development. It is likely that the elimination of some specific DNA sequences from the developing macronucleus may be the cause of the irreversible loss of the ability to undergo mitosis and meiosis, typical of the adult macronuclei of ciliates.

The question about the nature of the DNA which is lost from the macronuclear anlagen of *D. anser* presently remains open. The drop of the DNA content at the stage of 32-40 anlagen may mean genetic diminution, i.e., elimination of certain parts of the initial genome which would lead to a qualitative change of the gene complement of the adult macronuclei as compared with the micronuclei. However, not excluded is also the possibility that some "silent" (non-transcribed) or spacer DNA may be selectively eliminated from stage III macronuclear anlagen. Such a phenomenon could lead, e.g., to activation of the genes in the anlage. This question can be solved by investigating the biochemical properties of the DNAs of the micronucleus and of the macronucleus at various stages of its post-conjugational development.

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Etude en Microscopie Electronique a Balayage de la Theque de Qelques Thecamoebiens en Provenance de Trois Biotopes d'un Etang

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Synopsis. Dix espèces de Thecamoebiens caractéristiques de trois biotopes d'un même étang ont été observés en microscope élèctronique à balayage. La relation entre la structure de leur thèque et l'influence du milieu est mise en évidence. Certains microcaractères originaux pourraient avoir une importance taxonomique.

Les caractères taxonomiques utilisables facilement comme critères spécifiques chez les Thecamoebiens, concernent surtout la morphologie de la thèque et sa structure. Ce dernier caractère est à présent parfaitement illustré par le microscope électronique à balayage, qui permet de détailler la nature des éléments et leur disposition à la surface de la thèque, comme l'ont déjà démontré plusieurs études (Ogden et Hedley 1980, Ogden 1983, Netzel 1975, Coûteaux et al. 1979).

Dans cette étude, nous donnons des exemples de structures de thèques chez quelques espèces choisies parmi les plus communes, provenant de trois types de biotopes différents d'une même station.

L'analyse faunistique de cette station a été effectuée auparavant à l'aide du microscope photonique et a révélé la présence de 107 espèces réparties dans 10 Familles différentes. (Chardez et Gaspar 1976).

Matériel et méthode

Les échantillons proviennent de trois „niches écologiques" de l'étang des Sarrazines, situé dans le domaine Forestier des Epioux (Province de Luxembourg, Belgique). Les prélèvements se répartissement comme suit:

A — Sur les mousses immergées au bord.

B — Dans la zone de déclivité, à 1 m du bord, 20 cm de profondeur. (Sapropèle).

C — Dans la vase du fond au centre de l'étang à 2 m de profondeur.

Les espèces triées au microscope inversé, ont été lavées à l'eau bidistillée, puis collées sur supports en laiton, séchées et recouvertes d'une fine pellicule conductrice d'or au moyen d'un appareil à cathode crachante. Les examens ont été faits à l'aide d'un microscope Jéol JSM 35.

Observations

Biotope *A*

Parmi les Mousses immergées, les espèces les plus communes appartiennent à la Sous-classe des *Filosia*. Dans ce groupe, la thèque est formée d'écailles siliceuses d'origine endogène, dont la forme et la disposition varie d'après l'espèce.

Trinema complanatum Penard (Pl. I 1)

Ecailles circulaires, disposées sans ordre par superposition. Des écailles d'un diamètre plus petit sont réparties au niveau du pseudostome, une fine denticulation à pointes mousses entoure le bord du pseudostome.

Trinema enchelys (Ehrenberg) Leidy (Pl. I 2)

Ecailles circulaires, irrégulièrement imbriquées, avec des écailles ovales, plus petites au niveau du pseudostome. Certains interstices entre les grandes écailles peuvent être bouchés par de petites écailles ovales ou circulaires.

Le pseudostome est bordé de fines épines, leur longueur est irrégulière et d'environ 0,8 microns.

Tracheleuglypha acolla Bonnet et Thomas (Pl. I 3)

Ecailles circulaires régulières et régulièrement imbriquées, pseudostome simple, sans col, souvent ourlé par un léger vernis organique.

Euglypha tuberculata Dujardin (Pl. I 4)

Ecailles elliptiques ou ovalaires, régulières et régulièrement imbriquées, à la façon des tuiles d'un toit. Les écailles péribuccales sont denticulées, cette denticulation caractérise souvent les différentes espèces du Genre (Coûteaux et al. 1979).

Chez ces quatres espèces, on trouve deux modes de revêtement décrit par Thomas (1958): un mode de revêtement par imbrication complète par les seuls grands disques ne laissant aucun espace libre chez *Tracheleuglypha* et *Euglypha* et un mode où les grands disques laissent entre eux des espaces comblés par de petites écailles (cas de *Trinema*).

Biotope *B*

Dans le sapropèle de la zone de déclivité, les espèces de la sous-classe des *Lobosia*, deviennent plus communes.

Chez ces espèces, la thèque est constituée d'un vernis organique plus abondant, incorporant des éléments minéraux divers, souvent clairsemés, plaquettes polymorphes ou petites pierres, souvent mélangées à des Diatomées remaniées ou non. Parmi les espèces les plus abondantes dans nos prélèvements, nous citerons:

Centropyxis aculeata (Ehrenberg) Stein (Pl. I 5)

Chez cette espèce, le fond de la thèque est hérissé de cornes creuses, plus ou moins langues et en nombre variable.

Centropyxis sylvatica (Deflandre) Thomas (Pl. I 6)

Dans ce cas, la thèque est peu pierreuse et presque toujours incrustée de Diatomées d'espèces diverses.

Difflugia globulosa Dujardin (Pl. I 7)

Chez cette espèce, de nombreux spécimens ont la thèque entièrement recouverte de grandes Diatomées identiques, appartenant aux Genres *Nitzschia*, *Cymbella* ou *Achnantes*.

Certains Thecamoebiens ont l'habitude d'agglutiner les mêmes espèces de Diatomées. Ce caractère a été également observé chez: *Difflugia diatomosus* Chardez et Gaspar, *Centropyxis discoides* Penard, *Phryganella dissimulatoris* Chardez et *Difflugia urceolata suburceola* Chardez et Gaspar.

Biotope *C*

Dans la vase du fond de l'étang, vivent surtout des espèces de grande taille, dont la thèque est généralement constituée d'éléments quartzeux. Nous citerons ici quelques exemples parmi les plus communes dans nos prélèvements: *Difflugia pyriformis* Perty (Pl. I 8), *Difflugia corona* Wallich et *Difflugia elegans teres* Penard (Pl. I 10). L'ensemble

de la population possède une structure formée d'un mélange de plaquettes, d'éléments pierreux, de spicules de Diatomées et très souvent des kystes siliceux de Phytoflagellés (Chrysomonadines), (Pl. I 11). Nous avons rencontré cette particularité chez de nombreuses espèces: *Difflugia manicata* Penard, *Difflugia penardi* Hopkinson *Difflugia globularis* (Wallich) Leidy, *Difflugia lacustris* (Penar) Jung et *Centropyxis aerophila* Deflandre.

Conclusions

Cette étude nous a permis, grâce au microscope élèctronique à balayage, de préciser certaines caractéristiques morphologiques déjà entrevues au moyen du microscope photonique et de révéler certains détails originaux taxonomiquement interessants.

Les quelques exemples cités démontrent l'importance du milieu sur la structure de la thèque chez les Thecamoebiens, en particulier chez les espèces dont la thèque est formée et consolidée au moyen d'éléments exogènes empruntés sur place et qui caractérisent souvent le milieu d'origine.

SUMMARY

Ten species of testate *Amoebae* characterizing three biotops from the same pond were observed with a scanning electron microscope. The relationship between shell structure and influence of the environment has been shown. Some original microstructures may have a possible taxonomic importance.

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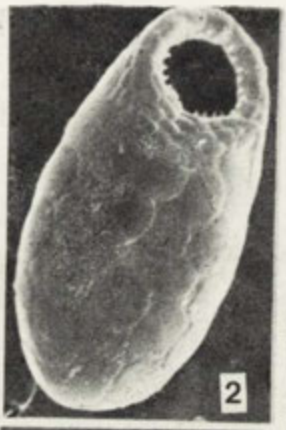
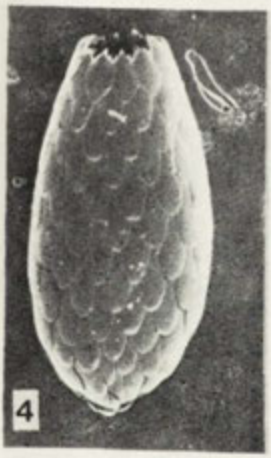
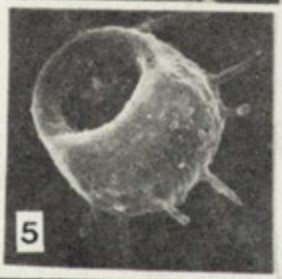
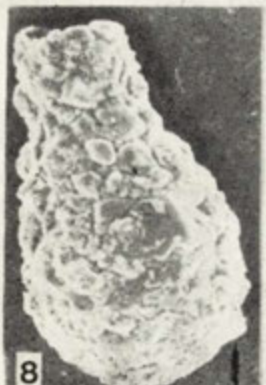
EXPLICATION DE LA PLANCHE I

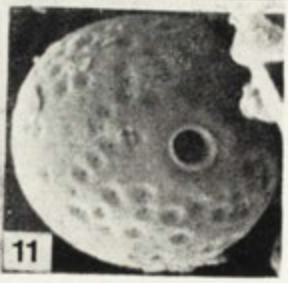
1: *Trinema complanatum* (× 3000)
2: *Trinema enchelys* (× 2600)
3: *Tracheleuglypha acolla* (× 1000)
4: *Euglypha tuberculata* (× 1200)
5: *Centropyxis aculeata* (× 440)
6: *Centropyxis aerophila* (× 940)
7: *Difflugia globulosa* (× 780)
8: *Difflugia pyriformis* (× 540)
9: *Difflugia corona* (× 300)
10: *Difflugia elegans teres* (× 600)
11: Kyste de Chrysomonadine (× 4000)











D. Chardez et A. Rassel auctor phot.

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Observations sur la Repartition des Thecamoebiens dans un Lac

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Synopsis. Dans ce travail, les Thecamoebiens (*Rhizopoda testacea*) ont été recensés dans un Lac de grande superficie, dans le but de contrôler la répartition des espèces dans les différentes zones écologiques constituant l'ensemble du biotope. 72 espèces ont été déterminées, se répartissant en deux populations caractérisant les berges et le fond. Trois espèces sont nouvelles pour la Science.

L'étude de la population thecamoebienne d'un Lac, considéré dans son ensemble comme un biotope, offre en réalité une série de niches écologiques où l'adaptation stationelle selon le concept de Deflandre (1937) joue un rôle important.

Les espèces colonisant les régions des berges sont généralement de petites tailles vivant habituellement au contact ou dans le voisinage des plantes aquatiques, tandis que les fonds abritent surtout des espèces de grandes tailles typiquement saprophiles.

Entre ces deux zones, il existe des régions intermédiaires où toutes les espèces s'interpénetrent et offrent souvent des populations hétéromorphes avec toutes ces variantes qui compliquent singulièrement leur déterminations. Il est bien connu que les espèces se divisent en séries d'unités systématiquement différentes (sous espèces, variétés ou formes) en liaison avec le milieu où certaines espèces présentes, subissent des changements et modifications qui les différencient sensiblement. Les avis des spécialistes sur les différences de dimensions par exemple, sont souvent différents.

Toutefois actuellement, on peut admettre qu'une "variété" dite "fixée" à le rang d'espèce, si elle possède une signification écologique et si ses caractères se transmettent héréditairement. Chez les Thécamoe-

biens, les caractères spécifiques les plus apparents, s'inscrivent naturellement sur la thèque, déterminant une sèrie de types morphologiques actuellement bien établis, comme conséquence directe du milieu (Bonnet 1975).

La présente étude, est un inventaire donnant la répartition et la composition des espèces de Thecamoebiens dans l'ensemble du Lac de Virelles, dont la particularité est une très grande surface pour une faible profondeur.

Le biotope

Le Lac de Virelles, situé dans la Province du Hainaut, en Belgique, est le plus grand plan d'eau d'origine naturelle du Pays. Sa superficie est de 130 Ha, la profondeur n'excède pas 2 m.

Le pH varie suivant l'endroit de 7,70 à 8,15.

Pour l'ensemble du Lac, nous avons considéré trois zones écologiques importantes.

A — La zone marginale, parmi les Roseaux (*Arundo sp.*).
Le sapropèle y est noir, très riche en matières organiques et peu minéral.
Prélèvements de 10 à 20 cm de profondeur.

B — La zone de déclivité, moins riche en matières organiques.
Prélèvements de 30 à 50 cm de profondeur.

C — Le fond du Lac, garni d'une épaisse couche de vase grise.
Prélèvements à plus ou moins 2 m de profondeur.

Méthode

Les 10 et 11 juin 1984, vingt échantillons de dépôts et d'eau d'une contenance de 100 cc ont été prélevés dans chaque zone considérée. Au laboratoire, les échantillons ont été placés dans des cristallisoires munis d'un aérateur. La recherche des Thecamoebiens se faisait par triage des espèces au microscope inversé.

Des préparations ont été montées dans l'Euparal.

Les mensurations maxima et minima étaient relevées pour chaque espèce.

Liste et répartition des espèces

Note: Les flèches indiquent le sens des contaminations et intercontaminations possibles entre les zones écologiques.

	Zones écologiques
Arcella gibbosa Penard	A→B
Arcella hemisphaerica Perty	A
Arcella hemisphaerica undulata Deflandre	A
Arcella rotundata Playfair	A→B
Arcella vulgaris Ehrenberg	A
Arcella vulgaris regularis Deflandre	A
Centropyxis aculeata [Ehrenberg] Stein	A↔B↔C

Centropyxis aculeata oblonga Deflandre	A
Centropyxis aculeata minima van Dye	A→B
Centropyxis aerophila Deflandre	A→B
Centropyxis constricta (Ehrenberg) Deflandre	B←C
Centropyxis marsupiformis obesa Deflandre	A→B↔C
Centropyxis ecornis turcestiana Chibisova	C
Centropyxis elongata (Penard) Thomas	A→B
Centropyxis marsupiformis obesa Deflandre	C
Centropyxis marsupiformis ecornis van Dye	C
Cyclopyxis arcelloides Penard	C
Difflugia acuminata Ehrenberg	C
Difflugia acutissima Deflandre	C
Difflugia acutissima giga Gauthier-Lièvre et Thomas	C
Difflugia acutissimella sp. nov.	B↔C
Difflugia amphoralis cornuta G. L. et Thomas	C
Difflugia beyensi sp. nov.	C
Difflugia bicruris G. L. et Thomas	C
Difflugia compressa (Leidy) G. L. et Thomas	C
Difflugia curvicaulis Penard	C
Difflugia corona Wallich	A↔B↔C
Difflugia cylindrus (Thomas) Ogden	C
Difflugia distenda Ogden	C
Difflugia difficilis Thomas	C
Difflugia gigantea Chardez	B←C
Difflugia glan Penard	C
Difflugia globularis Wallich	C
Difflugia gouttula Godeanu	C
Difflugia lacustris (Penard) Jung	A↔B↔C
Difflugia lobostoma Leidy	A↔B↔C
Difflugia lobostoma minor Chardez	A↔B↔C
Difflugia linearis (Penard) G. L. et Thomas	C
Difflugia labiosa Wailes	C
Difflugia lanceolata Penard	A↔B↔C
Difflugia maxilabiosa sp. nov.	C
Difflugia mamillaris Penard	C
Difflugia nodosa (Leidy) Chardez	C
Difflugia parva (Thomas) Ogden	C
Difflugia papillomata G. L. et Thomas	C
Difflugia pyriformis Perty	B←C
Difflugia pulex cuneata Playfair	A→B
Difflugia penardi (Penard) Hopkinson	A
Difflugia rotunda Ogden	C
Difflugia serbica Ogden et Zivcovic	C
Difflugia scalpellum Penard	C
Difflugia tricornis (Jung) Ogden	C
Difflugia urceolata minor Deflandre	C
Difflugia viscidula Penard	C
Difflugia ventricosa Deflandre	A
Difflugia venusta (Penard) Ogden	C
Zivcovicia compressa Ogden	C

Heleopera sylvatica Penard	A→B
Lesquereusia modesta Rhumbler	C
Plagiopyxis labiata Penard	A→B
Geoplagiopyxis declivus Chardez	A→B
Cyphoderia ampulla (Ehrenberg) Leidy	A
Cyphoderia ampulla papillata Wailes et Penard	A
Euglypha cristata Leidy	A
Euglypha laevis Perty	A
Euglypha rotunda Wailes	A→B→C
Euglypha tuberculata Dujardin	A
Tracheleuglypha acolla Bonnet et Thomas	A
Tracheleuglypha dentata (Vjedowsky) Deflandre	A→B
Trinema enchelys (Ehrenberg) Leidy	A→B
Trinema lineare Penard	A→B→C
Trinema lineare minuscula Chardez	A

A l'examen de ces listes, nous constatons que: la zone *A* contient 15 espèces caractérisant ce milieu, la zone *C* montre que 33 espèces sont caractéristiques du fond, tandis que 2 espèces qui peuvent être considérées comme erratiques ont été observées dans la zone intermédiaire *B*.

La contamination de *A* vers *B*, nous montre 12 cas, celle de *C* vers *B* seulement 2 cas, tandis que 8 espèces peuvent être considérées comme appartenant à la faune de dispersion générale (indiquées dans les listes par des doubles flèches).

Enfin, il nous semble important de préciser que: *Centropyxis aerophila, Euglypha rotunda, Trinema lineare, Trinema lineare minuscula, Plagiopyxis callida* et *Geoplagiopyxis declivus*, sont des espèces habituelles des milieux endogés et des Mousses corticoles, elles sont donc susceptibles de provenir des terres bordant le Lac.

Ces chiffres démontrent que la colonisation des Thecamoebiens s'effectue logiquement dès bords vers le fond, avec une faunule adaptée et caractérisant ces deux zones écologiques.

De très nombreuses thèques vides, n'entrent pas dans ces considérations, car après la disparition de l'Amibe, de nombreuses thèques finissent toujours par échouer sur les fonds.

Trois Genres apparaissent qualitativement et quantitativement riches: *Arcella, Centropyxis* et *Difflugia*. Les mensurations moyennes de l'ensemble de ces populations, nous donnent les chiffres suivants: Zone $A = 107$ µm, zone $C = 224$ µm; soit un peu plus du double. La zone *B*, est sans signification, car elle n'existe qu'en raison du mélange des espèces par intercontamination.

Ces données confirment d'autres observations (Penard 1905, Chardez et Gaspar 1982), à savoir, que les grandes espèces sont plus fréquentes dans le sapropèle des fonds que dans les régions de la beine et des berges.

Nombre d'espèces par Genre et par zone

Familles	Genres	Zones écologiques		
		A	*B*	*C*
Arcellidae	*Arcella*	6	2	0
Centropyxidae	*Centropyxis*	6	6	4
	Cyclopyxis	0	0	1
Difflugiidae	*Difflugia*	8	8	35
	Zivcovicia	0	0	1
Hyalosphenidae	*Heleopera*	1	1	0
Lesquereusidae	*Lesquereusia*	0	0	1
Plagiopyxidae	*Plagiopyxis*	1	1	0
	Geoplagiopyxis	1	1	0
Euglyphidae	*Euglypha*	4	1	1
	Tracheleuglypha	2	1	0
	Trinema	3	2	1
Cyphoderiidae	*Cyphoderia*	2	0	0

Notes sur quelques espèces

Difflugia acutissimella sp. nov. Pl. I 6, 9 et 10 b

Diagnose: Thèque allongée, renflée régulièrement de section circulaire, avec un pseudostome tronqué droit. Le plus grand diamètre de la thèque se situe aux environs du tiers antérieur, puis les flancs s'accuminent progressivement, pour former un prolongement caudal plus ou moins épais à bout fermé et arrondi, quelquefois légèrement incurvé.

Le revêtement est entièrement formé de petits grains quartzeux plats et peu débordants, régulièrement rangés au niveau du pseudostome. Ces éléments pierreux sont noyés dans un vernis organique hyalin à jaunâtre.

Mensurations: Hauteur = 100-126 μm. Diamètre = 46-58 μm. Diamètre du pseudostome = 22-24 μm.

Difflugia beyensi sp. nov. Pl. I 2-4

Diagnose: Thèque volumineuse parfaitement sphérique, régulière, surmontée d'un col court, cylindrique, assez étroit. Le pseudostome est bordé d'un épaississement régulier incrusté de très petits éléments pierreux. Le revêtement de la thèque est constitué d'éléments quartzeux polymorphes souvent peu débordants.

Mensurations: Diamètre de la théque = 257-277 µm, Diamètre du pseudostome = 83-85 µm, Hauteur du col = 39-48 µm,
Hauteur totale = 296-325 µm.

Etymologie: Le nom de cette espèce est dédié au Dr. L. Beyens.

Difflugia maxilabiosa sp. nov. Pl. II 12-15

Diagnose: Thèque ovoide, allongée, la partie postérieure est plus ou moins rétrécie, sans former une pointe, le pseudostome est circulaire, tronqué droit avec un très léger col sans lobe, le bord ne se retourne pas vers l'extérieur. La revêtement est formé de plaquettes minérales polymorphes et de petites particules pierreuses.

Mensurations: Hauteur = 350-363 µm, Diamètre = 264-277 µm, Pseudostome = 145-151 µm.

Note: Les préparations Types de ces nouvelles espèces, sont déposées dans les collections de la Faculté des Sciences Agronomiques de l'Etat à Gembloux, Belgique, sous les N° Y55, Y53 et Y54.

Difflugia serbica Ogden et Zivkovic, 1983

Nous avons observé cette espece dans sa forme typique conforme aux spécimens figurés par les Auteurs (1983). Pl. II 17 a-c, mais également des individus possédant une sorte de mammelon recourbé sur le côté. Cette espèces semble présenter plusieurs variantes de forme au niveau du fond de la thèque, dont le caractère principal est sa légère compression.

Difflugia pulex cuneata Playfair, 1917 Pl. II 11

Dans nos prélèvements, cette variété était plus allongée et plus régulière que le type figuré par Playfair [1917].

Hauteur: 40-46 µm, Diamètre: 15-25 µm, Pseudostome = 9-15 µm.

SUMMARY

The testate *Amoebae* have been recorded in a lake characterized by a large surface, in order to control the distribution of species in the various ecological zones composing the whole biotop. Seventy two species have been determined; they divide into two populations characterizing the banks and the bottom. Three species are new to science.

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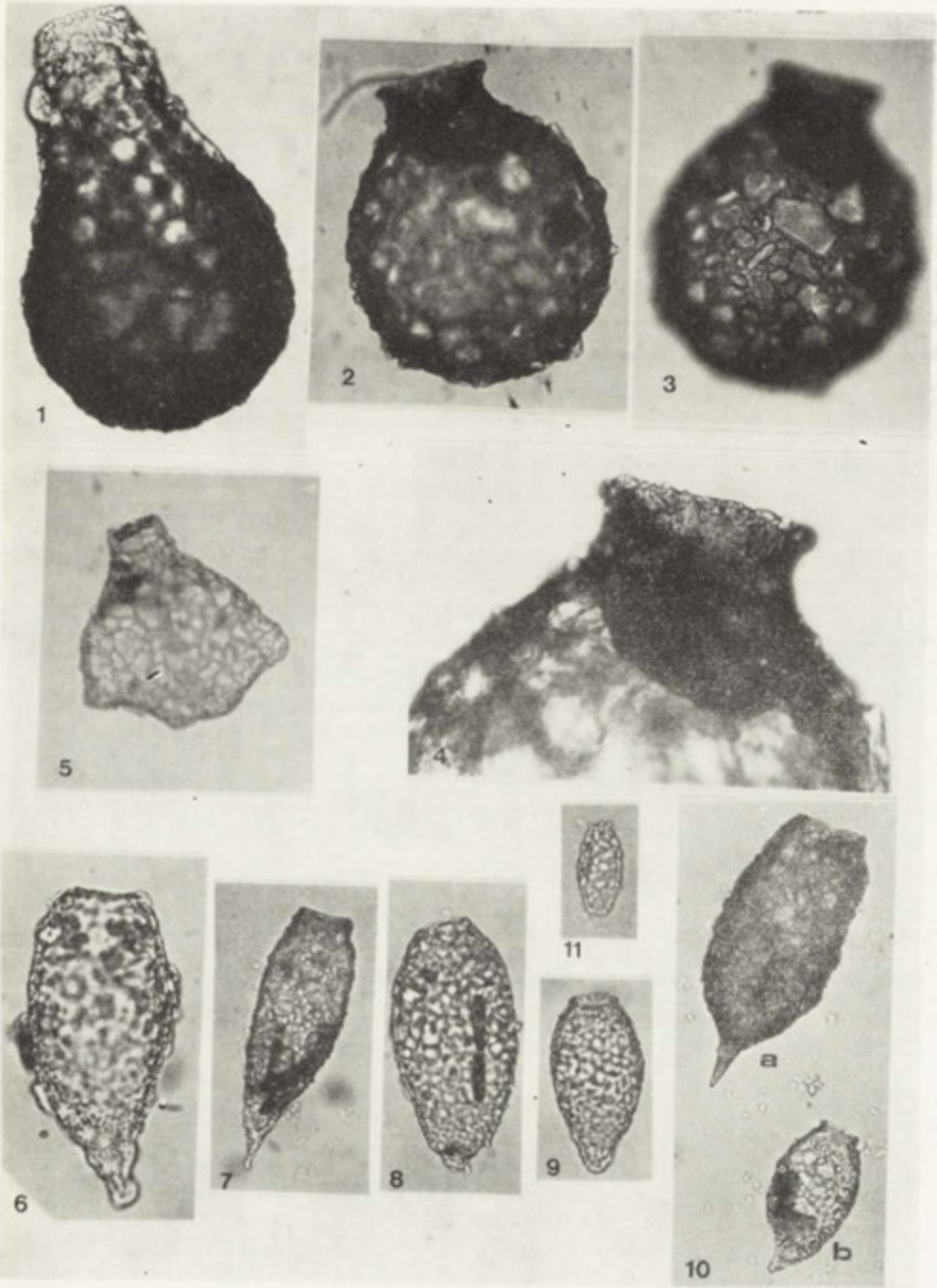
EXPLICATION DES PLANCHES I-II

1: *Difflugia gigantea* Chardez (× 200)
2 et 3: *Difflugia beyensi* sp. nov. (× 200)
4: *D. beyensi* sp. nov. (détail) du pseudostome (× 400)
5: *Difflugia nodosa* (Leidy) Chardez (× 200)
6 à 9: *Difflugia acutissilella* sp. now. (× 200)
10 (a): *Difflugia acutissima* Deflandre (× 100)
10 (b): *Difflugia acutissimella* sp. nov. (× 100)
11: *Difflugia pulex cuneata* Playfair (× 200)
12 à 14: *Difflugia maxilabiosa* sp. nov. (× 200)
15: *D. maxilabiosa* sp. nov. (détail du pseudostome (× 400))
16 et 17: *Difflugia labiosa* Wailes (× 200)
18: *D. amphoralis cornuta* Gauthier-Lièvre et Thomas (× 200)
19: *D. urceolata minor* Deflandre (× 200)
20: *D. bicruris* Gauthier-Lièvre et Thomas (× 200)
21: *Geoplagiopyxis declivus* Chardez (× 400)
22: *G. declivus* (détail du revêtement (× 600)



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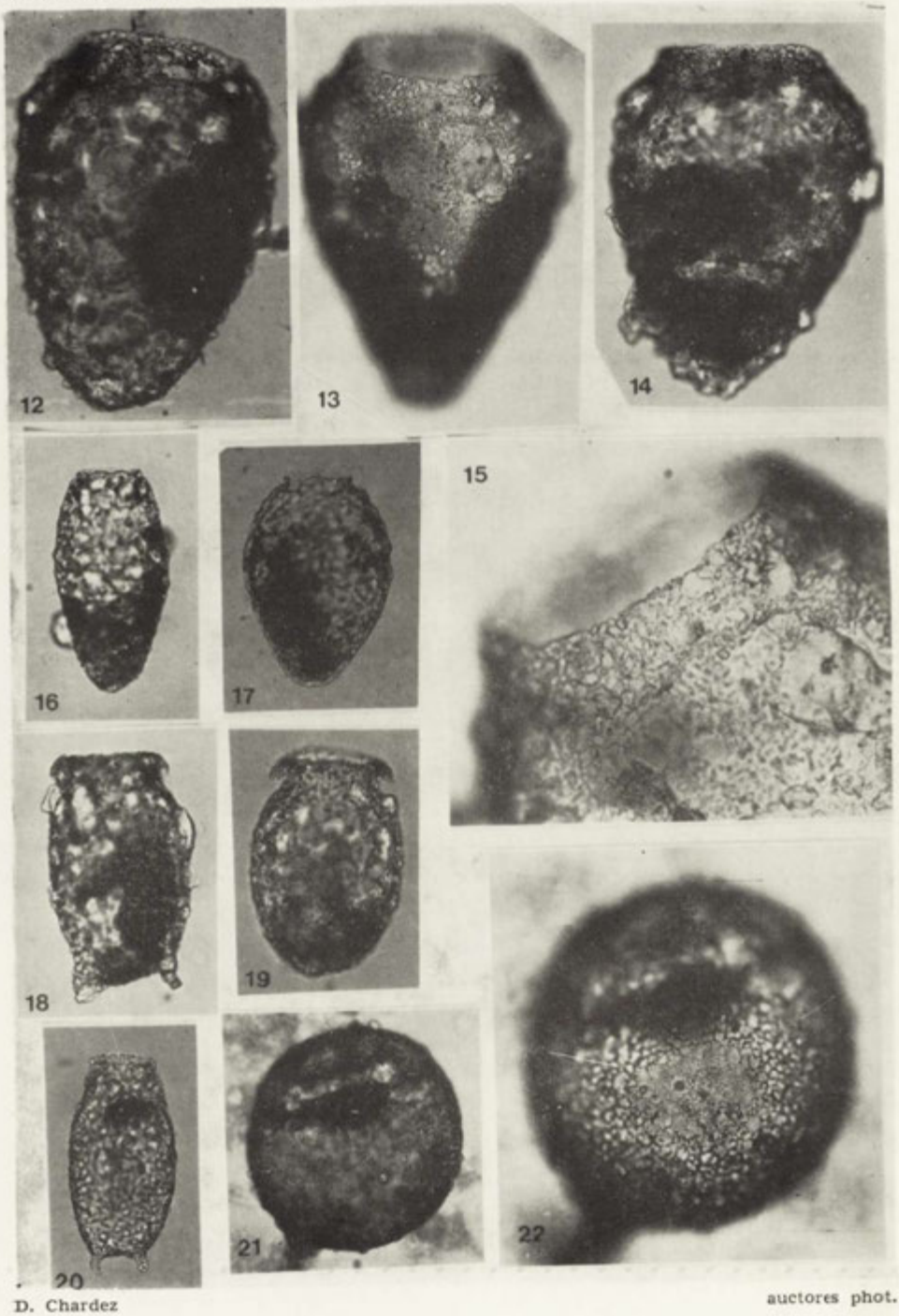

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Cytological Study of *Parastasia macrogranulata* Wita, 1984

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Synopsis. Parastasia macrogranulata Wita, 1984 — an euglenoid parasitic flagellate inhabiting alimentary tract of fresh water *Copepoda* has been investigated with the use of cytological methods at light microscope level. It has been proved that the life cycle of *P. macrogranulata* involves trophic phase (trophozoite) in the host gut and agamic reproductive phase in outer environment (in water). After leaving host gut trophozoites may transform into palmella-like forms and cysts. Cysts may persist long time, even more than three months. Morphology and functional activity of trophozoites, dividing and flagellate cells have been studied in details. Seasonal changes in the life cycle which ought to be taken into account in differential diagnosis and species identification are also recorded. *P. macrogranulata* appeared to be specific parasite of *Acanthocyclops bicuspidatus* Claus and *A. bisetosus* (Rehb.) in the environs of Leningrad.

First information about a parasitic euglenid *Parastasia macrogranulata* Wita, 1984 included the description of the trophozoite inhabiting the alimentary tract of fresh water copepods, as well as main data concerning its life cycle. However, complete life cycle of *P. macrogranulata* has been insufficiently known till now. So, the aim of the present paper was: (1) to recognize morphological particularities and functional activity of each stage of the life cycle using various cytological methods, and (2) to recognize ecology and seasonal modifications of the life cycle and to establish the list of hosts of this species.

Material and Methods

P. macrogranulata was found in the gut of copepods living in small ponds in the environs of Leningrad. Flagellates were removed from the gut and examined alive or in permanent preparations under the light microscope.

Observations of living flagellates were made in individual cultures (cf. Michajłow 1956, 1964). Microaquaria with parastasia taken out from a copepode, as well as infected copepods, were placed in a humid chamber in room temperature (20-25°C). Filtered water from the same ponds which copepods originated was used as culture medium.

Observations on the life cycle were made during all seasons of the year on living as well as on fixed material. For general morphology the flagellates were fixed either in Carnoy fixative or in absolute methanol following staining with (a) Mayer's hemalaun, (b) azur-eosin after Giemsa-Romanovsky and (c) hematoxylin after Carazzi. Staining with bromophenol blue or amido-black (Pearse 1968, Koročkin 1977) was also employed in order to examine the distribution of proteins in the flagellates. Mitochondria were revealed in preparations stained with acid fuchsin (Novelli 1959). Localization and shape of Golgi bodies were examined in the material impregnated with silver proteinate after Bodian or treated with OsO_4. Lysosomes were stained *in vivo* with 0.1% neutral red in aqueous solution (Bulychev et al. 1978, Bulychev and Veselkina 1983).

Feulgen reaction, methyl green-pyronine staining after Brachet, gallocyanin-chromalum method after Einarson and staining with 2% solution of toluidine blue after Brachet were used for detection of DNA and RNA (Pearse 1968). Histones were revealed by staining with heparin and alcian blue (Labelle and Briere 1971).

Lugol's iodine and PAS reaction after Hotchkiss were used for detection of polysaccharides of the glycogen type and paramylon. Acid mucopolysaccharides were stained with 0.1% alcian blue solution in 3% acetic acid according to Steedman and with 0.1% toluidine blue in 30% and 1% ethyl alcohol (Pearse 1968). Moreover, mucopolysaccharides occurring in glycocalyx were intravitally stained with 0.01% aqueous solution of alcian blue (Krylenkov et al. 1979).

Neutral lipids were revealed by the use of a saturated solution of sudan red III in 70% ethyl alcohol, while phospholipids were stained with sudan black B (saturated solution in 70% ethyl alcohol). Drawings of fixed and stained specimens were made with the use of camera lucida (objectives 40 × and 90 ×).

Results

Hosts

Main hosts of *P. macrogranulata* in the environs of Leningrad are *Acanthocyclops bicuspidatus* (Claus) and *A. bisetosus* (Rehb.). In *A. vernalis* (Fischer), *Eucyclops serrulatus* (Fischer) and *Cyclops vicinus* (Uljan.) these parasites occur rarely. Both main hosts live in ponds of eutrophic type, containing great quantity of organic substances (Rylov 1948). They feed on various organisms constituting microplankton and microbenthos of these reservoirs (Monakov 1976).

The material was collected near the coasts of the ponds with muddy bottom. The pH of water was 5.0-5.6. Prevalence of parastasia in cyclops varied from 1 to 10 per cents. Some cases of mixed infection by

P. macrogranulata and *P. fennica* were also observed. Intensity of infection was low, most frequently 1-5 trophozoites in a copepode gut. In laboratory cultures the intensity increased up to 100 specimens of various size (= various age). Prevalence of infestation in laboratory conditions was also higher reaching up to 100 per cent during all seasons of the year.

P. macrogranulata was found only in mature copepods, in males as well as in females. In younger stages no parasites were observed.

Life Cycle

Post-divisional flagellate forms, resulting of palintomic division, are the main infective stage in the life cycle of *P. macrogranulata* (Fig. 1). They fall into the host gut together with other organisms serving as food. In the host gut they lose the flagellum, intensively feed, grow and form paramylon grains (Fig. 2). In spring and summer mature trophozoites attain 40-60 μm of the body length, and 20-22 μm of width. Two modes of persisting of parasite population under unfavourable conditions (winter, desiccation of ponds in summer) have been observed: in host gut or in form of cysts in outer environment.

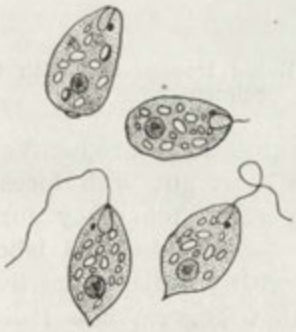

Fig. 1. Flagellate cells of *P. macrogranulata* (infective stage) descendants of palintomic division of one parental cell. Living specimens

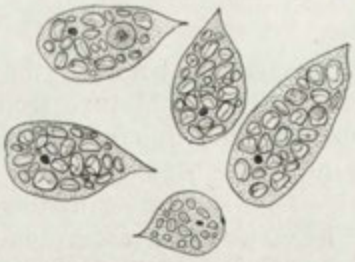

Fig. 2. Trophozoites of *P. macrogranulata* of various dimensions originating from the intestine of one *Acanthocyclops bicuspidatus*. Living specimens

(a) The life span of trophozoites in the host gut lasts 7-10 days in spring, summer and early autumn. In late autumn and winter, when the temperature of water decreases to 4°C the parastasia rest in the host gut. In this time the host usually is in anabiosis, burried in mud. Probably low temperature holds up palintomy and production of flagellate forms. In this time infection of new hosts does not occur. The trophozoites do not leave their hosts until spring, when temperature of water increases up to 15-18°C.

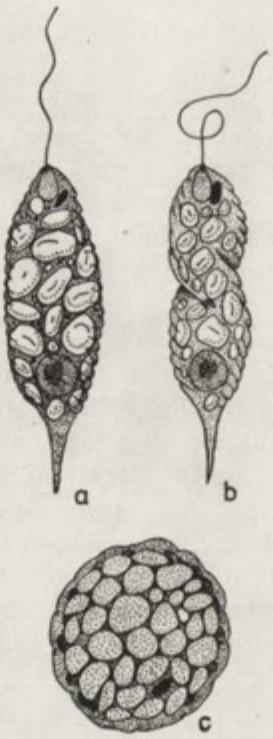

Fig. 3. *P. macrogranulata*, *a*, *b* — mature, free living trophozoites with the flagellum, *c* — cyst. Living specimens

After leaving host gut the protozoan begins the reproductive phase of the life cycle. Mature trophozoites leave host gut with feces; most abundantly they go out in spring. During 15-60 min they form the flagellum and begin to swim quickly (Fig. 3). In individual laboratory culture free swimming mature trophozoite with the flagellum lives 1-2 days in water (less mature ones live somewhat longer), then, it attaches to the substrate with its caudal processus, attains rounded shape, loses the flagellum and begins to divide (Fig. 4).

Fig. 4 *P. macrogranulata* trophozoite attached to the substrate before division. Living specimen

The trophozoite undergoes longitudinal division resulting in formation of two identical daughter cells. Nuclear division and greater part of the cytokinesis takes place in darkness (in night). So, at the early morning two daughter individuals, usually connected by a thin cytoplasmatic bridge (Fig. 5 *a*), may be seen. Soon, daughter cells divide

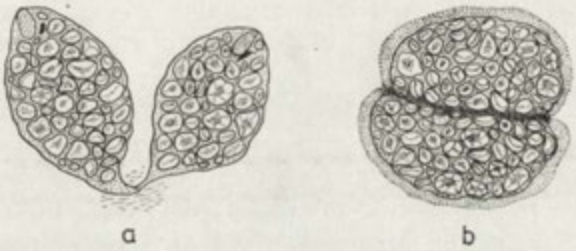

Fig. 5. Division of *P. macrogranulata* trophozoite. *a* — division without formation of envelope, *b* — division of palmella-like form (in envelope). Living specimens

once more, but the descendants do not achieve parental cell dimensions (palintomy). Division is achieved by formation of four daughter cells which separate from each other and begin to move due to slight metabolic contractions. After some time these cells produce flagella and transform into quickly swimming post-divisional flagellate forms (Fig. 1). Sometimes, the parental specimen divides only once producing two daughter cells and the second division does not follow. Most frequently formation of a temporary gelatinous envelope does not accompany the division of the parental cell, but sometimes such envelope may be clearly visible. In such cases the so called palmella stage is formed (Fig. 5 *b*).

Post-divisional flagellate forms actively swim in water during few days (3-5). During this time they ought to be eaten by copepods. In contrast to *P. fennica* the post-divisional flagellate cells of *P. macrogranulata* do not attach to the substrate but actively swim during the whole life span in water. This is an adaptation to food habits of their hosts, *Acanthocyclops bicuspidatus* and *A. bisetosus*. Both these copepods are predatory species feeding on microbenthic organisms (Monakov 1976). The post-divisional flagellates, not swallowed by copepods, attain rounded shape, lose flagella and die. At the end of free life in water paramylon grains are almost completely utilized and the cytoplasm becomes transparent.

The whole life cycle, including trophic and reproductive stages, lasts 10-15 days in spring, summer and early autumn. In winter the reproduction does not occur. In laboratory conditions, however, when the

temperature of water in the aquarium is maintained at about 20°C during the winter, the whole life cycle proceeds in the same way as in warmer seasons.

As the rule, only mature trophozoites go out from the host gut. Young specimens, not yet fully grown and having insufficient paramylon resources, do not produce flagella and do not divide. They survive only few days in water and die.

(b) Mature trophozoites, full of paramylon grains, are able to incyst in water. In the incysted stage they may survive long time (more than three months). Outer layer of the cyst is formed by a thin mucopolysaccharide sheath. Inside the cyst the stigma and large paramylon grains, characteristic of the species, are visible. The cyst is round, 30-40 μm in the diameter. It is infective for the hosts. After being swallowed by the cyclops the trophozoite liberates from the cyst and begin the parasitic stage of life. Cyst formation seems to be the main way of persistence of this species during unfavourable conditions.

Morphology and Functional Activity of Trophozoites

Mature trophozoite is cylindrical in shape, slightly narrowed and rounded at the anterior end, with tapered posterior end elongated into a sharply pointed tail (Fig. 3 *a*, *b*, Fig. 6). Body shape and dimensions vary depending on the stage of maturity of the trophozoite, and on the season of the year. In spring, summer and early autumn mature

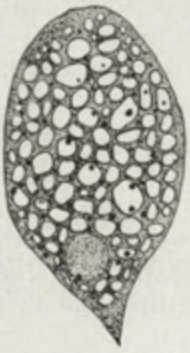

Fig. 6. Morphology of *P. macrogranulata* trophozoite from the host gut. Stained with Mayer's acid hemalaun with eosin

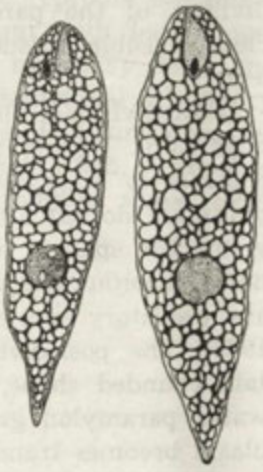

Fig. 7. Morphology of *P. macrogranulata* trophozoites from the host gut at the beginning of winter (December). Living specimen

trophozoites measure 40-60 μm in length and 20-22 μm in width. In the late autumn (October-November) the body dimensions increase up to 84 μm in length and 26 μm in width (Fig. 7). No trophozoite produces the flagellum during its life in the host intestine.

At the anterior body end a depression is situated leading to a flagellar reservoir, sphaerical or ovoid in shape. Changes of the shape of the reservoir depend on metabolic movements of the trophozoite. The stigma, composed of few granules orange in colour, closely adheres to the wall of the reservoir. The contractile vacuole is situated near the wall of the reservoir, opposite to the stigma.

Finelly striated pellicle covers the body of the trophozoite. The glycocalyx, including hyaluronid acid (Fig. 8), is well developed. Peripheral layer of the cell contains also proteins. In the subpellicular zone of the cytoplasm some muciferous bodies, characteristic of euglenids, are distributed.

Fig. 8. *P. macrogranulata* trophozoites. Vital staining with alcian blue. Glycocalyx visible

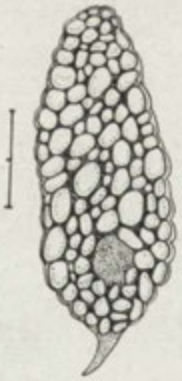

Fig. 9. Mature *P. macrogranulata* trophozoite stained with amido-black

The paramylon is the main structural and metabolic component of the cytoplasm. The shape and dimensions of paramylon grains is a characteristic feature of *P. macrogranulata*. Most grains are large, measuring 5-7 μm in length. Their shape is variable: sphaerical, ovoid, polyhedral or irregular (Fig. 3, Fig. 9). The lowest stock of grains occurs in young trophozoites, up to 20 μm of length (Fig. 2). During growth the quantity of paramylon in the cytoplasm increases proving that intensive synthesis of this carbohydrate takes place and that the trophozoite intensively feeds on account of its host.

Although at the end of the life span of intestinal forms the cytoplasm of mature trophozoites is filled with paramylon grains in all

seasons, especially high production of this substance occurs in late autumn, when the temperature of the host habitat falls down. In this period of the year the body dimensions of mature trophozoites increase and the body shape changes due to great number and dimensions of paramylon grains. The posterior part of the body undergoes the greatest change. It becomes thicker and the cytoplasm of the tail, transparent at the beginning, fills up with paramylon grains (Fig. 7). Great quantity of paramylon grains highly affects the mobility of the trophozoite and holds up metabolic contractions.

The cytoplasm of the parastasia includes also some quantity of polysaccharides of the glycogen type (Fig. 10 *a*) in form of small granules dispersed among paramylon grains. Acid mucopolysaccharides occur in the cytoplasm of mature trophozoites, being revealed by alcian and toluidine blue in form of small granules (Fig. 10 *b*, *c*). Acid mucopolysaccharides are distributed in spaces between paramylon grains; β and γ metachromasia prove about high homogeneity of these substances.

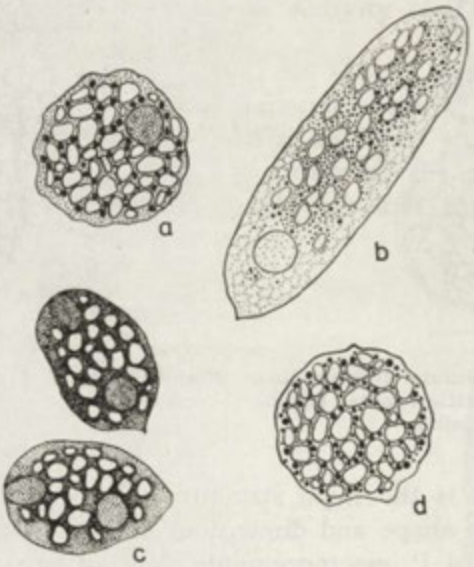

Fig. 10. Reserve substances in the cytoplasm of *P. macrogranulata*: *a* — PAS reaction, *b* — alcian blue after Steedman, *c* — toluidine blue, *d* — neutral lipids revealed by sudan red III

The content of lipids in the trophozoite cytoplasm is rather low. Small droplets of neutral lipids are located in all parts of the cytoplasm. Diffuse red colour obtained as a result of staining with sudan red III proves that the neutral lipids occur in diffuse state in the cell (Fig. 10 *d*).

Distribution of protein components in *P. macrogranulata* cytoplasm is correlated to high degree with the quantity of paramylon. The proteins are concentrated among paramylon grains in caudal part, in the nucleus, and also in the pellicle. Staining with heparin and alcian blue revealed histons in the nucleus of the trophozoites.

Mitochondria (Fig. 11) are spherical, ovoid or bacilliform bodies measuring about 1 μm. They are numerous, surrounding paramylon grains in all parts of the cytoplasm.

Lysosomes, 0.6-0.8 μm in the diameter are revealed by staining with neutral red (Fig. 12). They are dispersed throughout the cell, being especially numerous around the flagellar reservoir. They are primary as well as secondary. Strong development of the lysosomes proves that the cell feeds up intensively.

The Golgi apparatus formed of dictyosomes is situated in various parts of the cell. In preparations stained with OsO_4 separate dictyosomes are visible as well as groups of dictyosomes forming a chain surrounding paramylon grains (Fig. 13).

Fig. 11. Mitochondria in young *P. macrogranulata* cell. Stained after Novelli

Fig. 12. Lysosomes in mature *P. macrogranulata* trophozoite. Vital staining with 0.1% neutral red

Fig. 13. Dictyosomes (Golgi apparatus) in *P. macrogranulata* trophozoite after treating with 2% OsO_4

The nucleus of the trophozoite, spherical in shape, is situated behind the mid-length of the cell (Figs. 3, 6 and 7). It is 7-9 μm in the diameter and does not react with Feulgen reagent (Fig. 14). RNA is evenly distributed in the nucleus; it occurs also in great quantity in the cytoplasm. The amount of the cytoplasmatic RNA proves that high synthetic activity and quick transport of this substance from the nucleus to the cytoplasm takes place in the trophozoite.

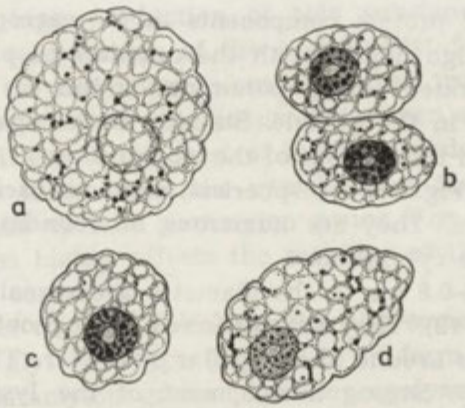

Fig. 14. Feulgen reaction in *P. macrogranulata* nuclei: *a* — mature trophozoite from host intestine, *b* — dividing trophozoite (in water), *c* — flagellate specimen (in water), *d* — young trophozoite from the host intestine

Trophozoites in the host gut do not divide resting in the interphase during this part of the life cycle.

Particular morphological characters of the diagnostic value of this species of *Parastasia*, based on detail investigation of the structure of mature trophozoites, living in water after leaving host intestine, are represented in the scheme (Fig. 15).

Agamic Reproduction

All stages of the agamic reproduction of *P. macrogranulata* take place outside the host body. Mature trophozoites, after leaving host intestine, differ from those resting in the gut only by presence of the flagellum and ability to swimming in water. The flagellum is about 50 μm long, being equal or almost equal in length to the body. The cytoplasm of mature trophozoite is filled with paramylon grains, so metabolic contractions are only feebly marked.

Division is preceeded by attachement of the cell to the substrate. The flagellum is thrown off, the amount of paramylon diminishes, and the nucleus intensively stains with Feulgen reagent, revealing chromatin aggregations. Predivisional specimens, ready to division, attain spherical shape; simultaneously the glycocalyx becomes thicker (Fig. 8).

As the result of the first palintomic division two identical daughter cells arise. Their nuclei are distinctly stained after Feulgen reaction (Fig. 14 *b*), showing well visible chromatin aggregations dispersed in the whole nucleus and central nucleolus.

After the second palintomic division four daughter cells are formed, identical by their morphology and dimensions but more than two times smaller from the parental cell. All descendant cells produce the flagella (Fig. 1) and transform into flagellate forms.

Flagellate Forms

Morphologically the postdivisional flagellate forms correspond to mature specimens being, however, smaller from the latter. Small amount of the paramylon makes their cytoplasm more transparent. The flagellum is equal in length to the body or slightly longer. The nucleus, distinctly stained after Feulgen reaction, shows chromatin aggregations and the nucleolus (Fig. 14 c). The cytoplasm contains fairly great amount of primary and secondary lysosomes giving evidence of intracellular utilization of the paramylon, the amount of which clearly decrease during free life in water (Fig. 1). If the postdivisional flagellate forms have no chance to get to the host intestine they die after utilization of the whole stock of the paramylon.

Discussion

Investigation on morphology and functional activity of *P. macrogranulata*, using various cytological methods, has shown that this colourless euglenid flagellate (Fig. 15) performs a complicated life cycle with alternation of endoparasitic and free living phases. *P. macrogranulata* is a heterotrophic organism comprising great amount of carbohydrates, mainly paramylon, in the cell. Pirenoids — centres of paramylon synthesis in many green euglenoids, in the cytoplasm of *Parastasia* have

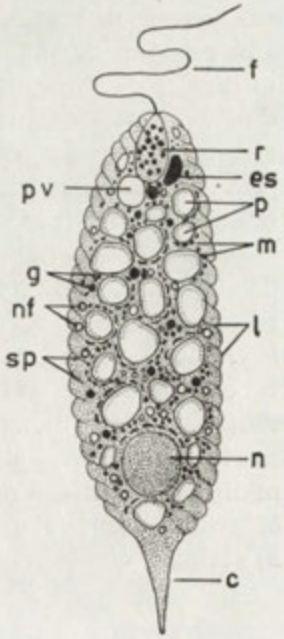

Fig. 15. Scheme of structure of mature *P. macrogranulata* trophozoite: *f* — flagellum, *r* — flagellar reservoir, *es* — stigma, *pv* — contractile vacuole, *p* — paramylon (grains), *m* — mitochondria, *g* — glycogen type polysaccharide, *nf* — neutral lipids (droplets), *l* — lysosomes, *n* — nucleus, *c* — tail, *sp* — pellicular striation

not been revealed. It is supposed that the synthesis of paramylon takes place at the membranes of dictyosomes as their distribution corresponds to the distribution of paramylon grains. The trophozoite cytoplasm contains fairly great amount of acid mucopolysaccharides, characteristic of euglenoids. A quantity of the polysaccharide of the glycogen type has been also discovered giving evidence of heterotrophy of the organism and its close relation to free living *Astasia longa* (Fig. 16).

Fig. 16. Polysaccharide of glycogen type in *Astasia longa* after PAS reaction

Situation of the nucleus in the trophozoite is one of the diagnostic characters. In mature trophozoite the nucleus does not stain after Feulgen reaction during its life in the host gut, as well as in the beginning of the free living stage. Negative result of Feulgen reaction may be due to particular functional activity of the trophozoite: in the host intestine it actively feeds, produces paramylon and other polysaccharides, proteins and subsequently RNA, necessary for production of a complex of enzymes. DNA in the trophozoite nucleus is dispersed.

The trophozoite cytoplasm contains considerable amounts of RNA deeply staining by pyronine and other specific stains. According to some records in the literature the nuclei of cells intensively synthetizing RNA do not stain after Feulgen reaction (Ringertz 1969, Krygier-Stojałowska 1975). Probably this is the case of *Parastasia*. During the palintomic division, when DNA is concentrated, the nuclei begin to stain intensively after Feulgen reaction and the aggregations of chromatin and central nucleolus are well visible inside them. The nuclei of postdivisional flagellate forms stain also intensively. The differences in staining of the nuclei after Feulgen reaction are especially interesting

for recognition of the cellular cycle of *Parastasia* using autoradiography and cytophotometry.

The trophozoites of *P. macrogranulata* show many adaptations to parasitic mode of life in the host intestine. These are: lack of the flagellum and euglenoid movement, occurrence of metabolic movement, due to which the parasites may stay in the intestine feeding on intestinal content, and accumulation of reserve substances necessary for subsequent phases of the life cycle. In the host gut the trophozoites do not divide. Agamic reproduction due to palintomy takes place in outer environment and differs greatly from that of *P. fennica* (Michajłow 1966, 1972, 1978, Wita and Sukhanova 1983). In *P. macrogranulata* two subsequent divisions give only four flagellate cells while in *P. fennica* palintomy results in production of 64-128 flagellate cells.

P. macrogranulata shows a daily rhythm of division characteristic of many photosynthetic euglenids.

In the reproductive phase of the life cycle the protozoan, after leaving host gut, adapts itself to another environment: a locomotory flagellum is produced, enabling active swimming in water, and the tail elongates serving for attachment to the substrate before division. The amount of paramylon is sufficient for the whole period of free life. Consumption of the paramylon, which may be observed during the whole period of life in water, proves that *Parastasia* does not feed during this time. Formation of palmella-like cells and cysts are also adaptative characters in the life cycle of *P. macrogranulata*. The cysts persist long time in the outer environment and in all seasons of the year; they are infective for copepode hosts.

The analysis of particularities in the life cycle proves that *P. macrogranulata* attained high level of plasticity in adaptation to various factors of the outer environment. For example, longevity of the life cycle changing in various seasons of the year, seasonal morphological variation characteristic of trophozoites and changes in a series of morphological characters (body dimensions, amount of paramylon grains) may exert a bearing on species identification. *P. macrogranulata* is a specific parasite of *Acanthocyclops bisetosus* and *A. bicuspidatus* and at all stages of the life cycle shows adaptative characters to the behaviour and habitats of these copepode species.

Comparison of structure of *P. macrogranulata* with other representatives of the genus having the stigma (Michajłow 1972, 1978, Wita 1978, 1984 a, b) shows that it is a separate species of colourless euglenoid flagellates parasitizing alimentary tract of *Copepoda*, with all characters of the genus *Parastasia* Michajłow.

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Autoradiographic Investigation of RNA Synthesis in the Macronucleus of the Ciliate *Nyctotherus cordiformis* at Different Stages of Its Life Cycle

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Synopsis. Incorporation of ^{3}H-uridine into the macronucleus of the ciliate *Nyctotherus cordiformis* was investigated at different stages of its life cycle, the time of incubation being 0.5, 1, 3, and 24 h. Detectable incorporation was observed at average after an incubation with tritiated uridine for at least 3 h. The macronucleus of *N. cordiformis* proved to synthesize RNA both in vegetative cells and exconjugants. In the latter, the fragments of the old macronucleus and the anlage of the new macronucleus at middle and late stages of its development were active in RNA synthesis. The macronuclei of encysted cells and young macronuclear anlagen were free of labelling. A local pool of RNA precursors is likely to exist around the vegetative macronucleus, the fragments of the old macronucleus and the macronuclear anlage.

Both kinds of nuclei of the ciliate *Nyctotherus cordiformis*, inhabiting the rectum of some species of frogs and toads, undergo rather complicated changes in the course of its life cycle. Conjugating ciliates occur only in the intestines of tadpoles just before the appearance of front legs; the post-conjugational nuclear reorganization may last as long as several weeks (Bojeva-Petruschevskaja 1933, Wichterman 1937, Golikova 1963).

The micronucleus (Mi) of either conjugant undergoes three pregamic divisions, giving rise to two pronuclei, the stationary and the migratory. After an exchange of the migratory pronuclei and their fusion with stationary pronuclei, the conjugants separate. The synkaryon undergoes one postzygotic division, and of the two nuclei arising one becomes

a Mi, and the other, a macronuclear anlage (Wichterman 1937). The chromosomes of the latter become polytenic (Golikova 1964). By the end of the first pregamic division of the Mi, the macronucleus (Ma) of either conjugant becomes irregular in outline, shifts posteriad from its usual place over the peristome, and at least gradually breaks into a large number of small round fragments.

The present investigation was aimed to establish the occurrence of RNA synthesis in the fragments of the old Ma of *N. cordiformis* during the period of nuclear reorganization. The existence of a rather long-lasting RNA synthesis in the fragments of the old Ma has already been demonstrated autoradiographically in exconjugants of *Paramecium aurelia* (Kimball and Perdue 1964, Berger 1973), *Euplotes woodruffi* (Rao 1968), *Bursaria truncatella* (Sergejeva 1976), *Didinium nasutum* (Karadzhan and Raikov 1979) and *Blepharisma musculus* var. *seshachari* (Dass et al. 1982). On the other hand, the activity of the Ma-fragments may be short-termed as well. In the course of conjugation of *Stylonychia mytilus*, the fragments of the old Ma incorporate RNA precursors for only one hour (from 5 h to 6 h after the onset of conjugation); long-living stable mRNA is synthesized in the old Ma during this period of conjugation (Sapra and Ammermann 1973).

Material and Methods

The ciliates, both vegetative cells and exconjugants, were collected from the intestines of tadpoles of *Rana temporaria* and *Bufo bufo* in the environs of Leningrad. The tadpoles had one or two pairs of legs. The ciliates were accumulated and washed in Ringer's solution for poikilotherms, diluted with distilled water to a half. Washed ciliates were incubated in the same medium with addition of ^{3}H-uridine (specific activity 9.99×10^{11} Bk/mmol, or 37×10^8 Bk/ml). Its activity in the medium was 2.22×10^8 Bk/ml. The ciliates were incubated at room temperature for 0.5 h, 1 h, or 3 h, and at 4°C for 24 h (the exconjugants of *N. cordiformis* have a rather low viability at room temperature).

After incubation, the ciliates were quickly washed with non-radioactive medium and fixed with Carnoy's fluid. Then, the ciliates were embedded in paraffin using Peterfi's method (the ciliates were previously attached to celloidine plates). Sections 5 μm thick were prepared and mounted on slides. After removal of the paraffin, the sections were air-dried. Some preparations were treated with 1% solution of crystalline RNase (Sigma) at pH 6.8 for 3 h at 37°C. The sections were covered with Ilford L-4 fluid emulsion diluted 1 : 3 with distilled water. The preparations were exposed for 30 days at 4°C. The radioautographs were developed in Kodak D-19 for 3 min. The preparations were then stained with methyl green-pyronin according to Unna-Pappenheim.

The silver grains over the sections were counted using an ocular grid with a 90× lens, eyepiece 7×. As the area of one fragment of the old Ma was smaller

at this magnifications than the area of a grid square (76.6 μm^2), the average amount of silver grains per one grid square was found for the fragments by calculation. For this purpose, the areas of 100 fragments were measured, the mean area of a fragment was calculated, and a coefficient (3.18) was introduced. The grain counts over one fragment were then multiplied by this coefficient to give the number of grains corresponding to a grid square.

Results

Incubation for 0.5 h

The mean number of silver grains over the Ma of vegetative cells after 0.5 h of incubation of the ciliates with tritiated uridine was only 1.4 per ocular grid square (76.6 μm^2), varying from 0 to 10 grains over individual sections of the Ma (Table 1). Most interesting is the

Table 1

Mean number and ±SD of silver grains over cell structures after incubation of *N. cordiformis* with ^{3}H-U (per one square of ocular grid at 90×7 magnification). Numerator — without, denominator — with previous RNase digestion, + signs indicate presence of a rim of silver grains around the nucleus, ++ signs — a dense two-layered rim

Type of cell	Cell structures	Time of incubation (h)			
		0.5	1	3	24
Vegetative cells	Cytoplasm	2.0±0.2 / 1.4±0.2	3.6±0.2 / 2.4±0.2	4.8±0.3 / 2.7±0.3	16.1±0.6 / 5.8±0.5
	Macronucleus	1.4±0.3 / 1.7	2.2±0.2 / 2.2±0.4	8.1±0.8 / 2.5±0.5	40.1±2.8 / 3.7±0.5
	Border of grains around the Ma	++ / ++	++ / ++	++ / ++	++ / ++
Exconjugants	Cytoplasm	2.7±0.2 / 1.5±0.2	4.0±0.2 / 2.2±0.2	6.9±0.4 / 2.5±0.2	11.4±0.5 / 2.8±0.3
	Fragments of old Ma — Per one fragment	0.6±0.07 / 0.5±0.1	0.6±0.05 / 0.5±0.07	6.3±0.4 / 2.8±0.4	24.0±1.6 / 1.6±0.3
	Fragments of old Ma — Calculated per grid square	1.8 / 1.5	2.1 / 1.6	20.1 / 8.9	76.0 / 5.1
	Fragments of old Ma — Border of grains around the fragments	++ / +	++ / +	++ / +	++ / ++
	Ma-anlage	6.4±0.4 / 2.8	3.4±0.4 / 1.5±0.2	15.6±1.3 / 17.0	6.4 / 10.6
	Border of grains around the Ma-anlage	+ / +	++ / +	++ / +	++ / +

fact that many silver grains were situated around the Ma, looking as a ring of 1 to 3 rows of grains (Pl. I 1). Over the cytoplasm, the number of grains was also low (at average, 2 grains per grid square, ranging from 0.5 to 5 in different ciliates). However, there were some local accumulations of grains (Pl. I 2), apparently over food vacuoles containing labelled bacteria. Often grains of silver were arranged along some lines into chains (Pl. I 3). The grains also marked the upper margin of the peristome, lying over the kinetosomes of the membranelles (Pl. I 4).

RNase treatment of the sections did not remove the label from the Ma and the cytoplasm (Table 1); in digested preparations the "border" of grains around the Ma was even more prominent (Pl. I 5).

In the exconjugants, the mean number of silver grains over one fragment of the old Ma was 0.6, while the means for individual specimens ranged from 0 to 3 grains per fragment. This corresponds to an average of 1.8 grains per ocular grid square (Table 1). As in the case of vegetative Ma, a border of silver grains occurred around some fragments (Pl. I 6). RNase digestion practically did not remove the label from the fragments, including the border around the fragments (Pl. I 7).

Silver grains were also seen over sections of Ma-anlagen at middle and late stages of their development, when they contain prominent polytene chromosomes (Pl. II 8). As compared with other cell structures, the Ma-anlagen were labelled heavier (over 6 grains per grid square, Table 1). Especially, a border of silver grains around the anlage was often prominent (Pl. II 9). The label in the anlage was partly removed by RNase digestion, but that in the border around it was not (Table 1).

Over the cytoplasm of exconjugants, the amount of silver grains was practically the same as over the cytoplasm of vegetative cells: 2.7 grains at average, ranging from 1 to 4 in individual cells. Very often the grains formed chains (Pl. I 6). There seems to be a slight reduction of the cytoplasmic labelling by RNase treatment (Table 1). There were cases of strong labelling of contractile vacuole (Pl. II 10).

As a rule, the cytoplasm of *N. cordiformis* (both vegetative cells and exconjugants) was more or less filled with grouped or scattered symbiotic bacteria. Even after short incubation, these seemed to be labelled also (Pl. I 7).

Incubation for 1 h

A prolongation to 1 h of the incubation of *N. cordiformis* in a medium containing ^{3}H-U only slightly affected the amount of silver grains over the vegetative Ma (Table 1); the intensity of labelling varied from

1 to 5 grains per ocular grid square in different ciliates. In addition, many silver grains surrounded the Ma (Pl. II 11). This girdle of grains became especially prominent after RNase treatment of the sections (Pl. II 12, 13).

The number of silver grains was slightly higher over the cytoplasm of vegetative cells incubated during 1 h as compared with those labelled for 0.5 h (Table 1). In individual ciliates the cytoplasmic labelling varied from 1.5 to 10 grains per grid square. Much of the cytoplasmic label proved to be RNase-sensitive, but chains of grains over cytoplasm always persisted after RNase digestion (Pl. II 13).

The labelling of the fragments of the old Ma remained slight (1 or 2 grains over one fragment in different exconjugants, Table 1). Sometimes the fragments were, however, surrounded by some grains (Pl. II 14). Silver grains could be seen over the polytene chromosomes in many Ma-anlagen (Pl. III 15, Table 1). However, the Ma-anlagen of young exconjugants were devoid of label (Pl. III 16).

Prolongation of the incubation time to 1 h induced some increase of the number of silver grains over the cytoplasm of the exconjugants (Table 1). Chains of grains like those described earlier were often seen (Pl. II 14). RNase treatment of the sections produced an approximatively twofold decrease of the labelling intensity of both the cytoplasm and the Ma-anlage (Table 1, Pl. III 17).

Incubation for 3 h

With this time of incorporation of ^{3}H-U, the amount of silver grains over sections of the vegetative Ma showed a further increase (Table 1). Some grains also formed a rim around the Ma (Pl. III 18), which was especially prominent after treatment of the sections with RNase (Pl. III 19). But now, after 3 h incubation with ^{3}H-U, many grains appeared over sections of the Ma itself (Pl. III 20). There were slightly more silver grains over the cytoplasm (Table 1). The labelling of the cytoplasm became weaker after RNase digestion and that of the Ma also decreased (Table 1).

Fragments of the old Ma in the exconjugants were surrounded with rims of silver grains (Pl. III 21); the labelling of the fragments themselves was much heavier than with shorter incubation times and ranged from 1 to 10 grains of silver over one fragment or about 20 grains per area unit. Rather densely labelled were also the growing polytene chromosomes in the Ma-anlagen (Pl. IV 22).

Also the cytoplasm of the exconjugants became more strongly labelled (Table 1). RNase treatment of the sections caused a net decrease

of the labelling intensity of the cytoplasm and macronuclear fragments (Table 1, Pl. IV 23). A prominent labelling of symbiotic bacteria was observed (Pl. IV 24).

Incubation for 24 h

Prolonged incubation of all stages of the life cycle of *N. cordiformis* with ^{3}H-U at 4°C brought about a drastic increase of the labelling intensity of both nuclei and cytoplasm (Table 1). In most cases, silver grains over sections of the vegetative Ma were impossible to count (Pl. IV 25). The grains were easier to count over tangential sections of the Ma (Pl. IV 26), but this obviously lead to underestimated values in Table 1. Sometimes the grains over the Ma formed round clusters or ringlets (which may correspond to nucleoli) and also some chains (Pl. IV 27-29).

Here also, RNase treatment did not prevent the silver grains from forming a rim around the Ma (Pl. V 30), but it now strongly decreased the number of silver grains over the Ma itself (Table 1). The labelling of the cytoplasm became more dense and a half of it was removed by RNase digestion (Table 1).

In exconjugants, the silver grains occurred first of all over fragments of the old Ma, where they often were too numerous to be counted (Pl. V 31, 32). However, the intensity of labelling was different in different fragments of the old Ma.

A significant amount of grains occurred over sections of larger Ma-anlagen (Pl. V 33), but young Ma-anlagen lacked many label (Pl. V 32, 34).

Over the cytoplasm, grains of silver were very often collected in chains. There were many clusters of grains over food vacuoles.

Some labelling remained in the Ma-anlagen after RNase digestion of the sections (Table 1). At the same time RNase treatment strongly reduced the labelling of both the Ma-fragments and the cytoplasm (Table 1, Pl. V 35).

The cysts of *N. cordiformis* were never labelled after any duration of incubation used (Pl. V 36).

Discussion

It has been demonstrated with the aid of autoradiography that the Ma of ciliates is the locus of RNA synthesis (Prescott 1960, 1962, Kimball and Perdue 1962, 1965). Different types of RNA are released from the Ma into the cytoplasm where they take part in protein synthesis.

The data of the present investigation are in good agreement with the conventional scheme: the Ma is, as usual, the main source of RNA of the cell. There are, however, some peculiarities which concern the rate of appearance of the label in the macronuclear RNA. The incorporation of radioactive precursors into the RNA of the Ma of *Tetrahymena* occurs in a very short time: thus, ^{3}H-cytidine appears in the Ma in 1.5 min, and in 15 min it can already be detected in the cytoplasm (Prescott 1962). However, some other data (Kaffenberger and Eckert 1980) show that ^{3}H-U incorporation into the Ma of *T. pyriformis* occurs only in 30 min. In *Paramecium aurelia* ^{3}H-U and ^{3}H-cytidine appear in the Ma and the fragments of the old Ma after 10 min of incubation (Kimball and Perdue 1964).

In contrast to that, the amount of silver grains over sections of the Ma and of the cytoplasm of *N. cordiformis* is insignificant after incubation with ^{3}H-U for 0.5 h and 1 h, the labelling of the Ma being even lower than that of the cytoplasm (Table 1). The labelling of the Ma becomes prominent only after longer periods of incubation (3 h and especially 24 h). Thus, the incorporation of labelled precursors into the RNA is slower in *N. cordiformis* than demonstrated for *T. pyriformis* and *P. aurelia*.

Treatment with RNase decreases the labelling of both Ma and cytoplasm only after an incubation of cells with ^{3}H-U for 24 h and sometimes after 3 h (Table 1). Owing to the fact that a rather important control was not performed in the present investigation, namely, no extraction of non-incorporated labelled precursors was done before coating the sections with emulsion, it is now possible only to suppose that this RNase-insensitive labelling corresponds to free ^{3}H-U. In this connection, most interesting is the presence of a rim of silver grains around the vegetative Ma, fragments of the old Ma, and sometimes around the Ma-anlage, which is never abolished by RNase treatment (Table 1, Pl, I 1, 4-6, Pl. II 9, 11-13, Pl. III 18, 19, 21, Pl. IV 23, Pl. V 30, 35). This border may correspond to a local pool of RNA precursors around these nuclei, possibly stored in some RNase-insensitive bound form other than RNA, rather than in form of free nucleosides or nucleotides. One can suppose that such labelled precursors are not likely to be incorporated into the RNA of the nucleus till the pre-existing stock of unlabelled precursors is utilized. Then, the difference in the rate of incorporation of radioactive precursors into the RNA between *N. cordiformis* and other ciliates would find its explanation. Some places of storage of the non-incorporated radioactive precursor seem to exist in the cytoplasm as well. Sometimes, silver grains are collected in a line over a dense layer of endoplasm above the upper margin of the peristome; this label is often RNase-resistant (Pl. I 4, Pl. III 19).

The amount of silver grains over sections of the vegetative Ma becomes very high after 24 h of incubation (Table 1, Pl. IV 25-29). And now, RNase digestion removes about nine-tenths of the label. Thus, a true incorporation of labelled precursors into the RNA of the vegetative Ma of *N. cordiformis* occurs only after incubation with ^{3}H-U for more than 3 h. The uneven distribution of silver grains over heavily labelled vegetative Ma may apparently be explained by stronger labelling of nucleoli and chromatin bodies.

No RNA synthesis in the Ma of cysts of *N. cordiformis* could be detected after any time of incubation with ^{3}H-U. The cytoplasm of encysted ciliates was also clean of silver grains (Pl. V 36). The possibility of the cyst wall being impenetrable for tritiated uridine, even at early stages of encystment, cannot be excluded.

As in the case of the vegetative Ma, the labelling of the fragments of the old Ma in the exconjugants is too weak after 0.5 h and 1 h of incubation with ^{3}H-U to indicate RNA synthesis; also the label cannot be removed with RNase. But after 3 h of incubation, the labelling of the fragments becomes significantly higher (Table 1), and at least a half of the label is now removed by RNase treatment. With 24 h of incubation the fragments become heavily labelled (Pl. VI 31, 32), and more than 90% of the label is RNase-sensitive (Table 1). Thus, our results confirm the presence of active RNA synthesis in fragments of the old Ma in the course of nuclear reorganization. The intensity of RNA synthesis in fragments of the old Ma appears to be rather high. This is not surprising because exconjugants of *N. cordiformis* are actively feeding, rapidly growing cells.

The materials of our previous investigation (Golikova and Nilova 1967) make likely the existence of some synthesis of DNA in fragments of the old Ma of *N. cordiformis*. These fragments thus appear to be quite active nuclei synthesizing DNA and especially RNA during the period of nuclear reorganization, until the new vegetative Ma is developed. Aged Ma-fragments gradually become resorbed in the cytoplasm in the presence of acid phosphatase (Sergejeva 1964).

The presence of RNA in both vegetative Ma and fragments of the old Ma of *N. cordiformis* was demonstrated cytochemically earlier (Golikova 1965). But cytochemical tests for RNA in the polytene chromosomes of the growing Ma-anlage were always negative. Thus, the appearance of silver grains over sections of the Ma-anlagen at middle and late stages of their development was unexpected (Pl. II 8, 9, Pl. III 15, Pl. IV 22, Pl. V 33). The labelling of polytene chromosomes of

the Ma-anlage was higher after a short incubation time (0.5 h) than that of any other structure of the cells, and this labelling was already RNase-sensitive (Table 1). This phenomenon apparently indicates that the stock of unlabelled RNA precursors in or around the Ma-anlage is smaller or more rapidly utilized than that of the vegetative Ma or of the fragments of the old Ma. Also the labelled border around the Ma-anlage, consisting of RNase-resistant material which apparently corresponds to stocked non-incorporated ^{3}H-U, was not always present or incomplete (Pl. II 9, Pl. III 15).

So it seems that some types of RNA are synthesized in polytene chromosomes of the developing Ma-anlage of *N. cordiformis*, along with DNA synthesis which was demonstrated earlier (Golikova and Nilova 1967). However, whereas ^{3}H-thymidine is more intensely incorporated into the forming polytene chromosomes of young Ma-anlagen, no RNA synthesis could be demonstrated in such anlagen with any time of incubation, and ^{3}H-U incorporation clearly increased with the age of the anlage.

The fact that Ma-anlagen of random age were used in the different experiments possibly explains the abnormally low labelling of the anlagen wih 24 h of incubation (by chance, they were almost all young). Another explanation of the slow or absent increase of labelling with incubation time could be the supposition that newly synthesized RNA would rapidly leave the anlage, this is also consistent with the absence of pyroninophilia of the anlagen.

Polytene chromosomes of developing Ma-anlagen of some other ciliates were found to synthesize RNA, e.g., in *Euplotes woodruffi*, *E. eurystomus* (Rao 1968, Rao and Ammermann 1970), *Chilodonella cucullulus* (Radzikowski 1973). No RNA synthesis was observed in the polytene chromosomes of the Ma-anlage of several hypotrichous ciliates: *Stylonychia mytilus* (Ammermann 1968), *Oxytrich* (Spear and Lauth 1976). However, contradictory results have been obtained by Alonso and Jareño (1974) who observed ^{3}H-U incorporation into the polytene chromosomes of the Ma-anlage of *St. mytilus*. These data are consistent with the occurrence of endogenic RNA-polymerases in the Ma-anlage of *St. mytilus* (Gaude 1981).

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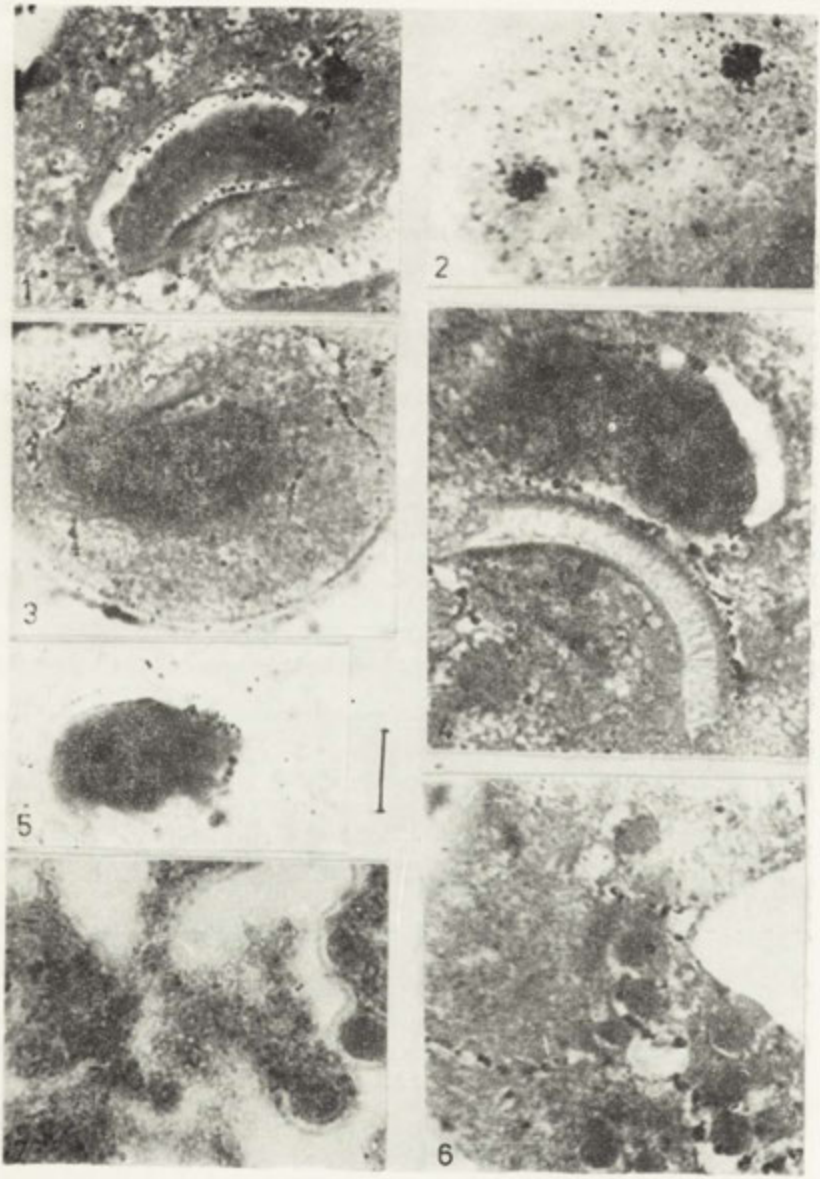
EXPLANATIONS OF PLATES I-V

Autoradiographs of sections of *Nyctotherus cordiformis* after incubation with ^{3}H-uridine: 1-10 — incubation time 0.5 h, 11-17 — incubation time 1 h, 18-24 — incubation time 3 h, 25-36 — incubation time 24 h. Scale for the plates — 10 μm

1: Border of silver grains over the vegetative Ma
2: Heavily labelled digestive vacuole
3: Chains of grains over the cytoplasm
4: Silver grains above the peristome and around the Ma
5: Border of silver grains around the Ma after RNase treatment
6: Silver grains around fragments of the old Ma and over the cytoplasm
7: Section of an exconjugant after RNase digestion
8: Labelling of polytene chromosomes of the Ma-anlage and of digestive vacuoles
9: Border of silver grains around the Ma-anlage
10: Accumulation of silver grains over the contractile vacuole
11: Silver grains around the vegetative Ma
12: Effect of RNase treatment on sections of the Ma: a definite border of grains around the Ma
13: Residual labelling of the cytoplasm and a rim of grains around the Ma after RNase digestion
14: Border of grains around fragments of the old Ma and chains of grains over the cytoplasm in an exconjugant
15: Labelling of polytene chromosomes of the Ma-anlage and of fragments of the old Ma
16: Unlabelled "young" Ma-anlage
17: Section of the Ma-anlage of the exconjugant after RNase treatment
18: Border of silver grains around the vegetative Ma
19: The rim of grains around the vegetative Ma after RNase digestion
20: Labelling of the vegetative Ma
21: Labelling of fragments of the old Ma
22: Labelled polytene chromosomes of the Ma-anlage
23: RNase-treated section of an exconjugant
24: Labelled symbiotic bacteria
25: Heavily labelled section of a vegetative Ma
26: Silver grains over a tangential section of the Ma
27-29: Uneven distribution of the label in the sections of the vegetative Ma
30: Border of silver grains around the Ma in RNase digested preparation
31, 32: Heavily labelled fragments of the old Ma
33: Labelled polytene chromosome of the Ma-anlage and chains of grains over cytoplasm
34: A "young" Ma-anlage without label (at arrow)
35: Effect of RNase treatment of an exconjugant
36: Section of a cyst — unlabelled



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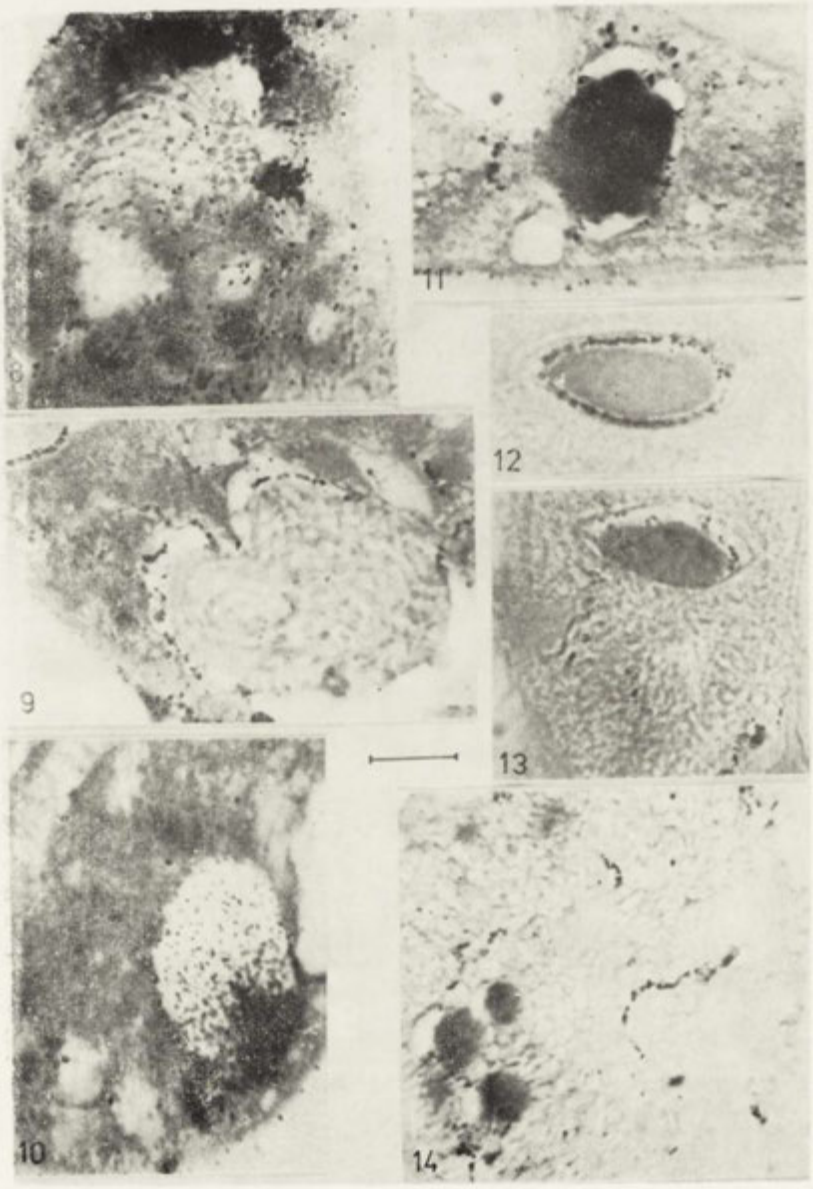

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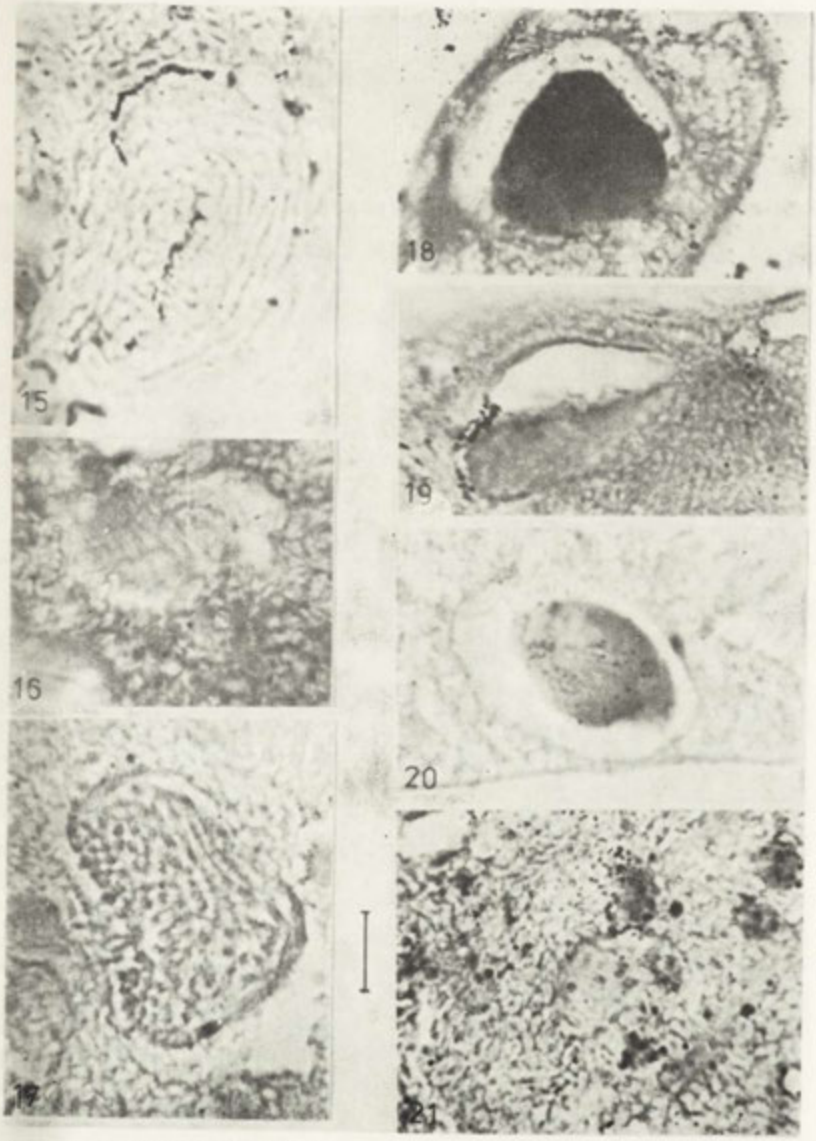

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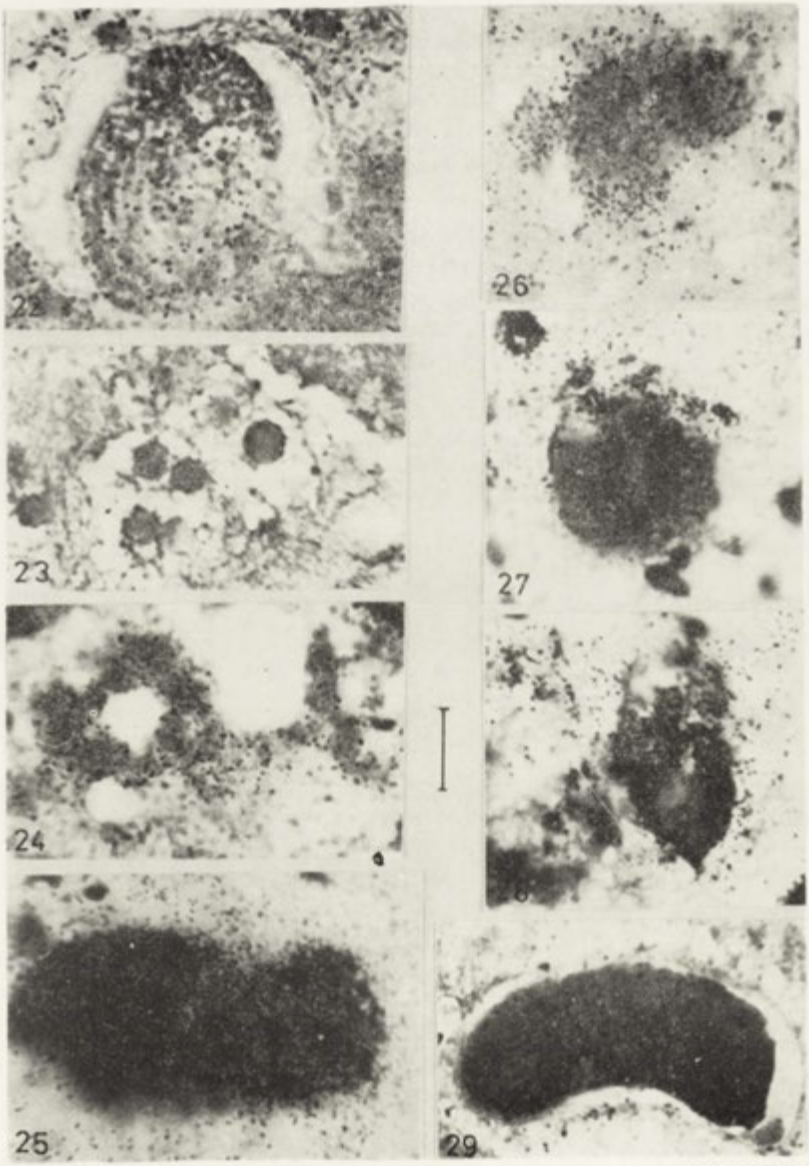

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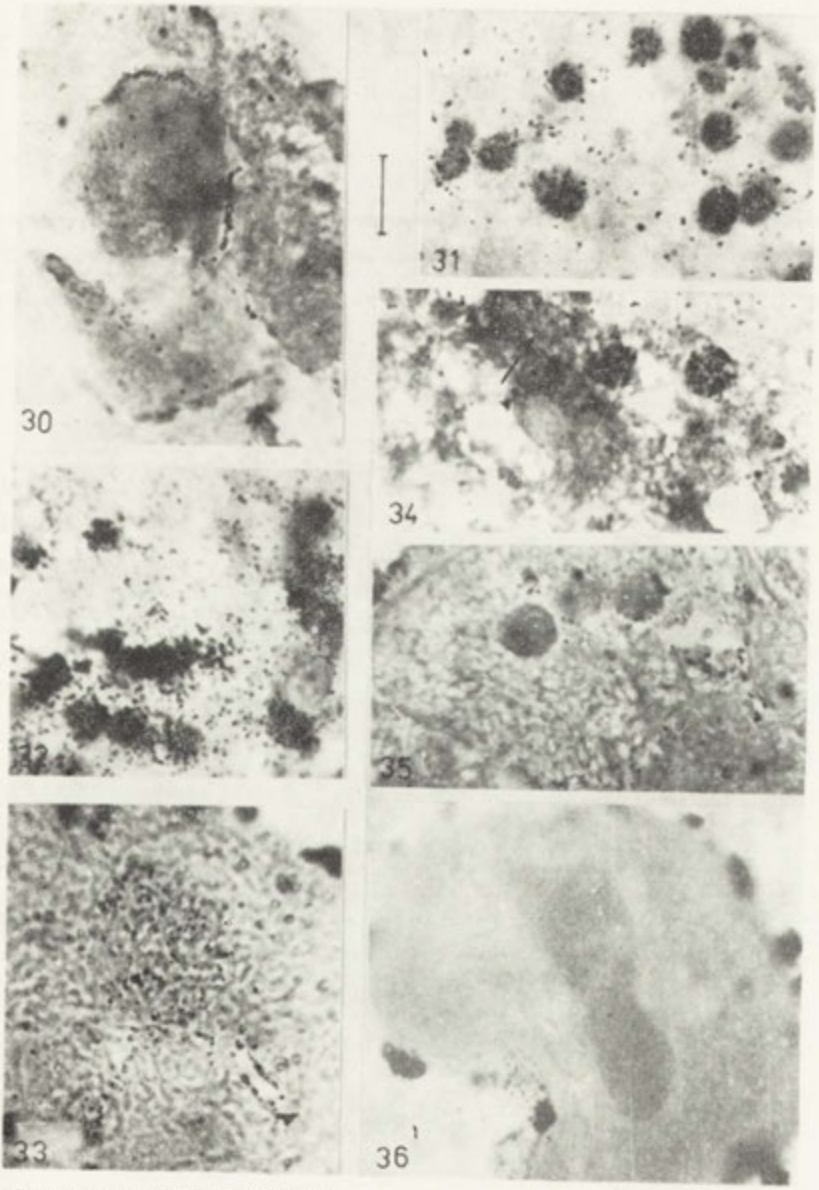

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Universality of Hormonal Imprinting in Different Taxa of *Tetrahymena* and Inter-strain Variations in Its Intensity

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Synopsis. Seven *Tetrahymena* taxa preexposed to insulin showed a quantitative change in binding potency upon reexposure to the hormone. This suggests that hormonal imprinting can be induced in all tested taxa of *Tetrahymena*. Imprinting was most efficient in the taxon having the lowest growth rate, whereas least efficient in the taxon with the highest growth rate among all strains studied. Lectin (Concanavalin A) binding of the various *Tetrahymena* taxa was different, with tendencies similar to insulin. Although insulin imprinted the cells for lectin binding, too, this effect was lower than for insulin.

The first interaction of the hormone with the cell gives rise to hormonal imprinting, which accounts for an altered — usually increased — cellular response to reexposure (Csaba 1980, 1981). In higher (vertebrate) organisms, the cell — hormone interaction, including receptor formation under hormonal influence, is genetically predetermined (Lin and Becker 1983). In unicellular organisms, which do not possess an encoded receptor — hormone system, hormone receptors may arise either from receptors for other materials, which originally represent non-specific structures for the hormone, or by assembly of certain membrane-associated subunits (Koch et al. 1979). Thus hormonal imprinting can also take place at the unicellular level (Csaba 1980, 1981). For example, the *Tetrahymena* possesses certain membrane structures which

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act as adequate receptors for hormones and can usually also mediate a specific response to these. The *Tetrahymena* is therefore an ideal model organism for investigations into the mechanism of hormonal imprinting (Csaba 1984, 1985).

Our hormonal imprinting studies had formerly been based on *Tetrahymena pyriformis* GL strain. In the present study we examined seven taxa of *Tetrahymena* for response to a vertebrate hormone (insulin), to clarify whetre hormonal imprinting was a potential of the GL strain in particular, or of all *Tetrahymena* species in general. We also studied the possible impact of sexual and asexual development on lin), to clarify whether hormonal imprinting was a potential of the progeny generations. Finally we pursued a possible interrelationship between cellular (membrane) lipid composition and intensity of hormonal imprinting, since indications of that have merged from earlier experiments (Kovács et al. 1984) with *Tetrahymena pyriformis* GL cells.

Materials and Methods

The taxa *Tetrahymena pyriformis* WH 14, GL (Zeuthen, Copenhagen), GL (Budapest), W, ST and Saphiro, as well as *Tetrahymena thermophila* and *Tetrahymena pigmentosa* (from Dr. Nanney) were grown in enriched proteose-pepton medium at 28°C. One-day cultures were used in the logarithmic phase of growth. Each mass culture was divided into two lots. One lot was treated with 10^{-4} M insulin (0.144 IU/ml Insulin Semilente, Novo, Copenhagen) for 1 h, and was subsequently returned to plain medium for 1 day, whereas the other lot was transferred to plain medium for one day without insulin treatment. After 24 h, the cell cultures were fixed in 4% neutral formaline, washed, and incubated for 1 h in presence of fluorescein-isothiocyanate (FITC) labelled insulin (BDH, England, FITC protein ratio: 0,14). Certain taxa were incubated for 1 h in the presence of FITC-labelled Concanavalin A (Con-A, Serva, FITC-protein ratio: 1.32). After incubation, the cells were washed in three changes of PBS, spread on slides, and assayed for intensity of fluorescence with a Canon Canola SX 320 apparatus, coupled with an Olympus MMSP fluorescence microscope. Thirty cells were assayed in each lot, and 3 replica assays were performed to calculate mean values, which thus covered 90 cells. The inter-group differences were analyzed for significance with Student's t-test.

The growth rates of the strains were determined in subcultures obtained single-cell cloneing in capillary tubes.

Results and Discussion

The FITC-labelled insulin was also bound by the cultures not pre-exposed to insulin, (Fig. 1). The binding capacity varied between taxa, being lowest in *T. thermophila* and highest in *T. pigmentosa* cultures.

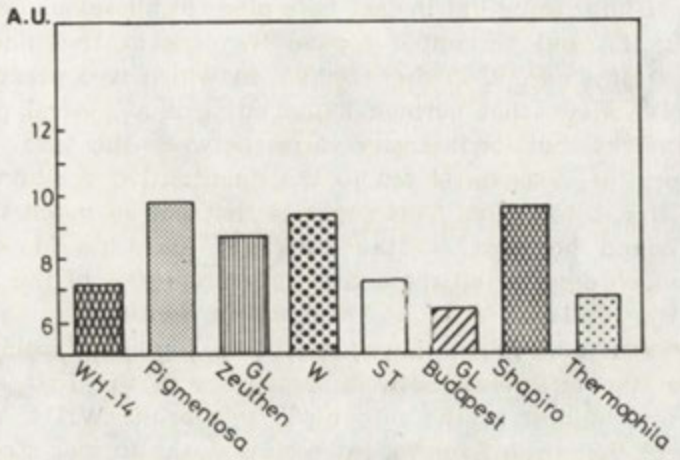

Fig. 1. Binding of FITC-labelled insulin to the different taxa of *Tetrahymena*

Although the difference between the two extremes approximated 50%, no parameter has been available for the quantitative evaluation of inter-strain variations in binding. No information emerged either on variations in the specificity of binding. These aspects were therefore disregarded, since the purpose of the study was *ab ovo* to assess the impact of the first interaction with the hormone (imprinting) from quantitative differences between binding relations on the first and second insulin exposure.

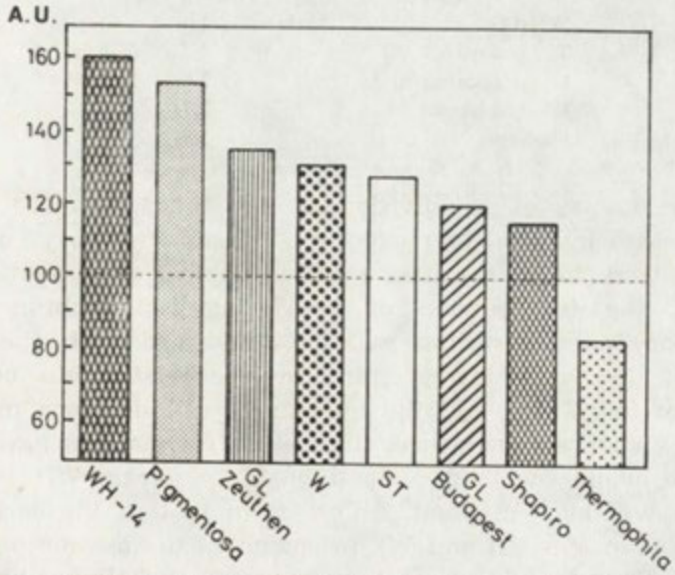

Fig. 2. Binding of FITC-labelled insulin to *Tetrahymena* pretreated (imprinted) with insulin. Values related to the not pretreated control as 100

Hormonal imprinting did in fact take place in all seven *Tetrahymena* species (Fig. 2), and accounted for an increase in the binding capacity of all strains except *T. thermophila*, in which it depressed affinity to insulin. It follows that hormonal imprinting is a general potential of the *Tetrahymena*, but its intensity varies between the taxa. The intensity of imprinting was unrelated to the quantitative relations of binding in the first interaction. This suggests that not so much the quantity of bound hormone, as the quality of the first interaction, which depends on the endogenous properties of the membrane, represents the decisive factor of hormonal imprinting.

The efficiency of imprinting (increase in binding capacity), as inferred from the difference between responses to the first and second exposure, was highest in the case of *T. pyriformis* WH14, and lowest in the case of the strain Saphiro. Interestingly, the former strain showed the lowest, whereas the latter the highest growth rate among all strains studied (except the negatively imprinted *T. thermophila*) (Table 1). There are two alternative explanations for the inverse relationship between the intensities of hormonal imprinting and growth rate: (1) The

Table 1

Growth rate of five (positively imprinted) *Tetrahymena* taxa related to the most slowly dividing WH-14 as 1

Taxon	Growth rate
WH-14	1
T. pigmentosa	2.5
GL (Copenhagen)	2.6
GL (Budapest)	2.7
Shapiro	5.6

greater the number of cell divisions in unit time, the more distant cell generations are involved in reexposure, and the "memory" of hormonal imprinting tends to decline after a certain number of generation changes, and (2) if the transmission of imprinting is membrane-associated, the "memory" of imprinting will be diminished with the increasing number of divisions. However, the inverse relationship between the intensity of imprinting and the number of cell divisions may as well as not be a chance coincidence, since other correlations have also been found. The membrane lipid composition of the strain WH 14, in which imprinting was most efficient, differs from that of the earlier studied *Tetrahymena* strains (Gl and W), inasmuch as the membrane of WH 14 contains a considerably greater amount of cardiolipin, linoleic acids

(n-18 : 2 $\Delta^{9,12}$; n-18 : 3 $\Delta^{6,9,12}$) and unsaturated fatty acids (Fukushima et al. 1978, Holz and Conner 1973). The membrane lipid composition has a considerable influence on membrane fluidity (Nozawa 1980) and membrane receptor formation (Dave and Witorsch 1983), and thereby on the imprinting mechanism as well (Kovács et al. 1984). It appears that differences in membrane lipid composition could account for quantitative differences in imprinting, and probably also in the mitotic rate.

The intensity of imprinting was unrelated to the sexual or asexual nature of the developmental cycle, i.e., to presence or absence of a micronucleus. Transmission of imprinting to the progeny generations did equally take place in sexually and asexually developing *Tetrahymena* strains.

Mention should be made of the experimental fact that the intensity of imprinting also differed between the Copenhagen and Budapest cultures of *Tetrahymena pyriformis* GL. It appears that the quantitative relations of imprinting may vary between different cultures of the same strain, depending on their origin, conditions of maintenance, and/or on the conditions of imprinting itself.

In addition the binding of Con-A to *Tetrahymena* was studied, as this lectin can occupy the binding sites of insulin (inhibiting the binding of this latter) without influencing the cells functionally (Csaba et al. 1983). In the present experiments Con-A bound to *Tetrahymena* in each taxa studied, and the sequence of binding capacity was very similar to those of insulin (Fig. 3). This support the earlier observations on the

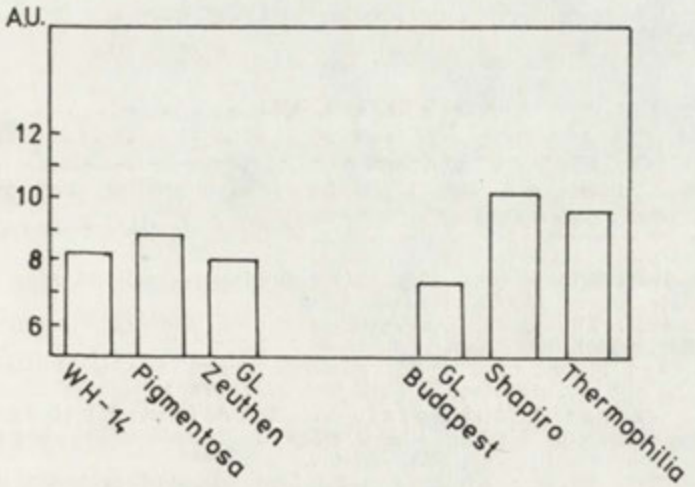

Fig. 3. Binding of FITC-labeled Con-A to the different taxa of *Tetrahymena*

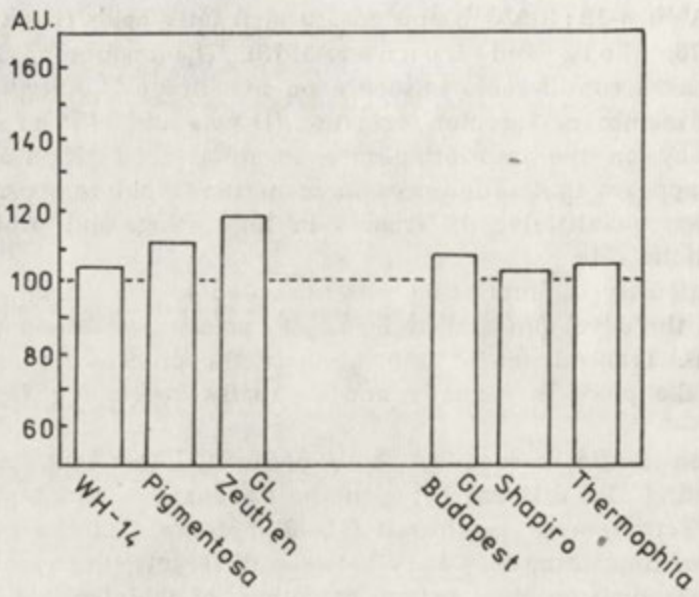

Fig. 4. Binding of FITC-labelled Con-A to *Tetrahymena* pretreated (imprinted) with insulin. Values related to the not pretreated control as 100

common binding site (Csaba and Kovács 1982, Sandra et al. 1979). Nevertheless, the imprinting for Con-A was much lower, than for insulin (Fig. 4), which shows the specificity of imprinting and demonstrates, that binding of Con-A is only a "side-product" of this latter. There was not similarity in the sequence of lectin binding capacity compared to insulin binding in the different taxa, demonstrating the development of sensitivity for insulin and Con-A after insulin imprinting.

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Effect of Inhibitors of Protein Synthesis and Endocytosis on Hormonal Imprinting in the *Tetrahymena*

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Synopsis. Methylamine, which inhibits clustering in the process of receptor-mediated endocytosis, did not interfere with insulin-induced hormonal imprinting in the *Tetrahymena*. Dimethylsulphoxide (DMSO) had no appreciable influence on imprinting by insulin either. However, hormonal (insulin) imprinting was inhibited by cytochalasine B, which acts on the microfilament system, by colchicine acting on the microtubular system, by the lysosomal enzyme inhibitor chloroquine, and by the Ca-inhibitor Ni^{2} as well. Among agents acting at transcription-translation level, actinomycin D and cycloheximide inhibited imprinting, whereas puromycin did not. In this light is appears that the inhibition of imprinting may differ between the levels of binding and functional response. Another conclusion emerging from the present study is that injury of the membrane of the *Tetrahymena* by inhibitors persists long in the progeny generations.

Hormonal imprinting, whose mechanism is the subject of this study, is essentially a far-reaching change which occurs at the first interaction of the hormone and its target cells, and accounts for an increased responsiveness of the cell to the given hormone throughout the further stages of cell life (Csaba 1981). Imprinting also alters the hormone binding capacity and certain functional states of the cell, and is demonstrable not only in those cells directly involved in the first interaction, but also in their progeny generations (Csaba et al. 1981, 1984). Several details of the phenomenon termed as hormonal imprinting are

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still obscure, but evidence has been accumulating on the important role of membrane receptors in the establishment of imprinting. Since the membrane receptors actively participate in certain transmembraneous transport processes, they occur not only in the cellular membrane, but also inside the cell, which they enter via endocytosis in coated vesicles, and become thus involved in recirculation processes which result in their reincorporation into the membrane. Thus the membrane receptors practically form a chain of information between the cell-coating membrane, and the cytoplasmic intracellular spaces (Brown et al. 1983, Fehlman et al. 1982, Steinman et al. 1983). The information transfer furnished by the membrane receptors is a highly complex process, which is controlled by several cellular-level factors. It follows that, presumably, imprinting is not limited either to membrane-level processes, being rather the issue of a chain of events.

The better understanding of the interrelated, viz. interacting events involved in imprinting can be facilitated if inhibitors are used to "blot out" one or another important cellular function, to learn about its involvement or non-involvement in the mechanism of hormonal imprinting.

In the living world, the key hormone of cellular metabolism is insulin, which can induce imprinting not only in multicellular organisms, but also in unicellular ones (Csaba 1980, 1981). We therefore used *Tetrahymena pyriformis* as model cell in the experiments whose description is forthcoming.

Material and Methods

Tetrahymena pyriformis GL cells, maintained in 0.1% yeast extract containing 1% Bacto trypton (Difco, Michigan) medium at 28°C, were used in the logarithmic phase of growth. The 24-h cultures were treated with 10^{-6} M insulin and/or with the following inhibitors, at the final concentrations specified: chloroquine (Chinoin, Budapest), 25 μmol : methylamine-HCl (Merck, Darnstadt, FRG), 10 μmol; cytochalasine-B (EGA-Chemie, Steinheim, FRG), 5 μg/ml; dimethyl sulphoxide (DMSO, Reanal, Budapest, 0.5%; DMSO also serves as solvent of cytochalasine-B); colchicine (BDH; Poole, England), 10 μmol; $NiCl_2$ (Reanal, Budapest), 1 μg/ml; cycloheximide (Koch-Light, Colnbrook, Bucks, England), 20 μg/ml; actinomycin D (Merck-Sharp-Dohme, N. J., USA), 3 μg/ml.

Paired samples were set up for parallel treatment with insulin + inhibitor and inhibitor alone, and further to the absolute control series an insulin-treated control group was also set up.

After treatment the *Tetrahymena* cells were returned to plain medium for further 24 h, were fixed in 4% neutral formaline, and washed in two changes of phosphate buffer (PBS 0.05 M phosphate buffer, pH 7.2; 0.9 M NaCl). Insulin

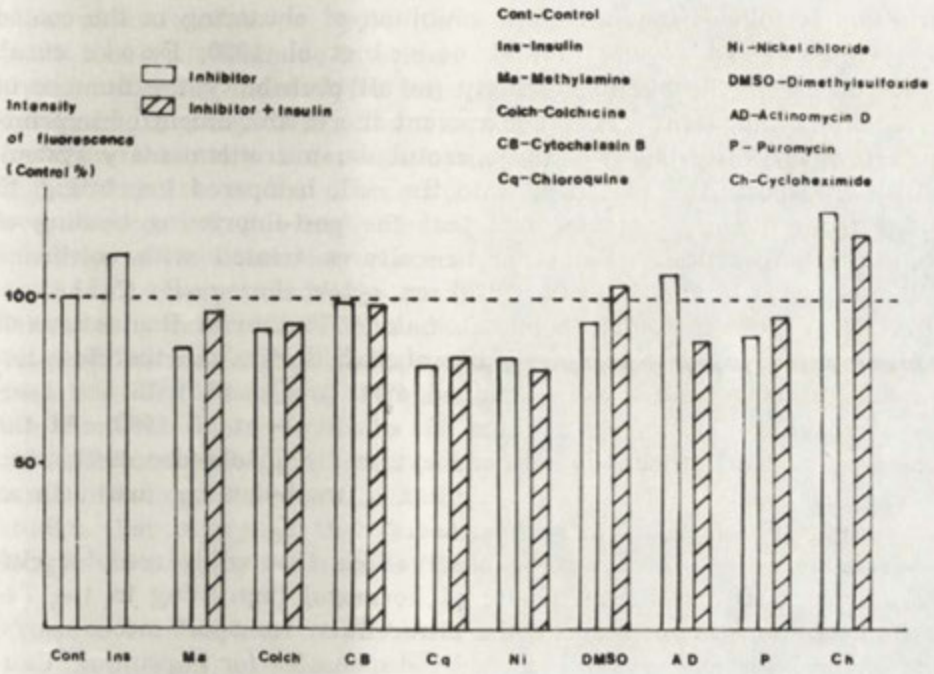

Fig. 1. Binding of FITC-insulin to *Tetrahymena* cells 24 h after pretreatment with insulin alone, inhibitor alone, or inhibitor + insulin, relative to the control as 100

binding was assessed by cytofluorimetry, after 1-h incubation of the samples in presence of fluoresceine-isothiocyanate. The results were evaluated with a HP 41C calculator, connected with the cytofluorimeter.

Twenty cells were assayed for fluorescence in each group, and 5 replica assays were performed with each sample, to calculate mean values for 100 cells in each series.

Results

We demonstrated earlier that methylamine and actinomycin D depressed, whereas colchicine did not influence, the imprinting effect of diiodotyrosine (T_2), as assessed from changes in the mitotic rate. All three inhibitors depressed, however, cell division rate induced by T_2 without imprinting (Csaba et al. 1982).

Part of the inhibitors used in the present experiments interfere somehow with endocytosis, whereas the other part with the transcription-translation processes. The inhibitors acting on endocytosis influenced imprinting to different degrees and by different modes of action. Methylamine depressed the binding of insulin, but did not fully abolish im-

printing. It follows that although inhibition of clustering in the coated pits (Maxfield et al. 1979, Michael et al. 1980, Poole et al. 1976) decreases the binding capacity (in all probability the number of receptors), it apparently does not prevent the establishment of imprinting. However, disturbance of the microtubular-microfilamentary system, which transports the coated pit into the cell, hampered imprinting, to judge from the experimental fact that the post-imprinting binding of insulin was practically the same in cultures treated with colchicine (Wunderlich and Speth 1970) or cytochalasine B (Nilsson 1977) as in those treated with inhibitor alone. The minor fluctuations of plus or minus trend were not significant statistically, and therefore negligible. Similar results were obtained after treatment with the lysosomal enzyme inhibitor chloroquine (Steinman et al. 1983). At the same time DMSO, which inhibits endocytosis by an obscure mechanism (Nilsson 1976, 1977), behaved similar to methylamine, inasmuch as imprinting did take place in its presence.

It follows from the foregoing observations that while receptor clustering is no essential prerequisite of hormonal imprinting in the *Tetrahymena*, normal operation of the intracellular transport mechanism(s) and of the lysosomal system is in fact indispensable for imprinting. Caution should be, however, exercised in interpreting this statement, since both colchicine and cytochalasine B act on the *Tetrahymena* in a manner slightly different from their action on other organisms. For example, colchicine does not damage the ciliary and cortical tubules (Wunderlich and Speth 1970), and cytochalasine B inhibits only the formation of nutrient vacuoles, but not that of pinocytotic vacuoles (Nilsson 1977). Moreover, the conclusions drawn above apply exclusively to the binding of the hormone, which may not necessarily show a parallelism with the functional response to it. For example, colchicine appeared to be indifferent for the *Tetrahymena* — and for cultured mammalian cells as well — when imprinting was assessed by impact on mitosis (Csaba et al. 1982).

Of the inhibitors acting at transcription-translation level, actinomycin D (Peters 1976) did not usually inhibit the binding of insulin, but inhibited the induction of imprinting by that hormone, as a rule to such degree that labelled insulin binding was even lower than after inhibitor treatment alone so that the inhibitor effect appeared to be potentiated by the hormone.

The two protein synthesis inhibitors differed remarkably in action. While puromycin inhibited the binding of insulin to the cell, but did not inhibit imprinting, cycloheximide enhanced rather than inhibited binding, but hampered the imprinting action of the hormone. At present

state of knowledge we could hardly explain this phenomenon. It appears that cycloheximide interferes with the very event of protein synthesis, which is essential for imprinting but non-essential for receptor synthesis, to judge from the circumstance that cycloheximide developed no inhibitory action in itself, since in its presence binding was highest relative to the other experimental series, and increased even over the control; this high binding value showed a minor decrease, if insulin was simultaneously present (imprinting).

Since 1-h exposure to the inhibitor or inhibitor + insulin had been followed by return to plain medium for 24 h, and insulin binding was assessed thereafter, it is obvious that the *Tetrahymena* cells had in the meantime divided several (at least 4-5) times, whence the effect of the inhibitors came into display in the progeny generations. In these, nearly all inhibitors tested accounted in themselves for a decrease in labelled insulin binding. This substantiates the conclusion emerging from earlier studies that injury of the *Tetrahymena* membrane (receptor) persists long in the progeny generations (Csaba et al. 1982).

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A Comparative Study of Chloramphenicol (CAP) Action on Resistant Mutants and on Wild-type Strains of Several Species of Two Ciliates *Hymenostomatida* (*Tetrahymena* and *Colpidium*)

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Synopsis. This paper reviews the effects of chloramphenicol on wild-type as well as on a set of mutant resistant strains belonging to several species of ciliates. The detailed analysis carried out in *Tetrahymena pyriformis* shows that the drug acts on the mitochondrial protein synthesizing system and that the mutations affect the mitoribosomes and are coded by mitochondrial DNA. Most of the other ciliate species (*T. rostrata*, *T. vorax*, *Colpidium campylum* and *C. kleini*) behave similarly to *T. pyriformis*.

Chloramphenicol like a number of other antibiotics is known to be an inhibitor of prokaryotic, chloroplastic and mitochondrial protein syntheses. This last synthesis has been previously studied with erythromycin on the ciliate *Paramecium* in our laboratory (Sainsard et al. 1974, Stelly et al. 1975, Perasso and Adoutte 1974). Consequently, these antibiotics have been useful tools to measure the quantitative and qualitative contribution of mitochondria to their own biogenesis.

In this summary, we compare some of our previously published data, obtained (with CAP treatments) on mutants and sensible cells of *Tetrahymena pyriformis*, with those obtained on other species of *Tetrahymena* in axenic conditions, and on *Colpidium* grown in monoxenic cultures which are unpublished data. All studied mutants are spontaneously selected in our laboratory.

I. Material and Methods

A. Studied strains

(1) Two strains of *T. pyriformis*

— Strain S. T., amicronucleate cells sensitive to CAP (originated from Pr. Suyama's laboratory, Philadelphia, USA)

— Strain D. 1968-5 (micronucleate cells sensible to CAP) and the resistant mutant denominated CA 103, isolated from this strain. Roberts and Orias (1973) had established by genetic experiments that its CAP resistance is cytoplasmically transmitted.

(2) *T. rostrata*

Micronucleate cells have been rendered sterile by passage through a "Stone's and Reynolds' pipette", (strain selected from the ciliate collection of Pr. Fauré-Fremiet, Orsay).

(3) *T. vorax*

Micronucleate cells (strain from the culture collection of Cambridge, England).

(4) *Colpidium campylum* (strain from the ciliate collection of Pr. Fauré-Fremiet).

(5) *Colpidium kleini* (strain obtained from cells isolated from a holestone of the campus, Orsay).

B. Culture Media

The various species of *Tetrahymena* grow in a P. P.Y.E. axenic medium (proteose-peptone 1.5%, yeast extract 0.25%) or in the defined medium of Rosenbaum et al. (1966). These cultures are always inoculated with 0.5 to 2% of a 24 h exponential growth culture of *Tetrahymena* (final dilution for growth curves counting: 1000-2000 cells/ml).

Colpidium grows in a dry lettuce decoction inoculated, 24 h before use, with *Enterobacter aerogenes.*

C. CAP Treatment

The antibiotic is dissolved at 80°C, in a stock culture solution after sterilization. The tested doses are from 50 to 500 µg/ml. The CAP action is studied during 24, 48 or 72 h.

D. Specific Methods

They will be exposed at the beginning of each chapter.

II. Selection of Spontaneous Mutants

A. Methods

— Cultures in 20 ml test-tubes with 250 µg/ml of CAP in the medium.

— Isolation and cloning of the eventual resistant cells some days after the inoculation of the sensible cells in this medium. Growth in 250 μg/ml CAP medium.

B. Results

Almost, if not all, cells die, and very few living cells have been observed after 8 days of culture.

We have isolated the following selected mutant strains:

— In the ST strain of *T. pyriformis*, STR_1, STR_2, STR_4 and STR_5 from four experiments with 250 μg/ml CAP medium STR_3 was isolated from 150 μg/ml CAP medium.

— In *T. rostrata* and *T. vorax* strains, one mutant strain of each from 250 μg/ml CAP medium.

No sensible cells were found in the species *Colpidium campylum* and *C. kleini*, even with the dose of 500 μg/ml CAP.

III. Experiments of Transplantation in *Tetrahymena*

A. Methods

A microquantity of cytoplasm of a resistant cell (STR_1 or CA) is injected in the cytoplasm of a sensible cell (ST or DN) placed in 250 μg/ml CAP medium. This delicate operation is performed on numerous cells and their behaviour is studied during several days.

B. Results

(1) The resistance takes a long time to appear because the mitochondria injected (or having spontaneous mutation factor) must multiply, in the presence of the selective CAP pressure in the medium, following the decrease of sensible mitochondria. Cells become black, and move slowly; when the injection is successful, cells can divide after 5-6 days, when their cytoplasm becomes clear and resistant.

(2) With 500 μg/ml CAP no resistant cell can be obtained with *T. pyriformis*: cells are not able to survive and die before having time to express their mutation capacity against the antibiotic (Perasso et al. 1980 a).

IV. Growth Pattern and Cell Morphology

A. Methods

— Cultures are made in Erlenmeyer with 100 ml medium containing different tested doses of CAP inoculated with about 1000 cells/ml (less for *C. kleini* which is bigger).

— Growth curves and generation time are deducted from counted cells. Cells of *C. kleini* are handly counted, the others are counted using a culture counter.

— Cell morphology is controlled by usual fixations for electron microscopy. Negative staining of mitochondrial cristae with PTA is used.

B. Results

(1) On Growth

— CAP inhibits the growth of sensitive cells and decreases the resistant cell's one

— Sensitive cells die in several days with 100 μg/ml

— The resistance of different strains in the same species is different for the same dose (250 μg/ml).

(2) On Morphology

The mitochondria of resistant and sensitive cells are smaller, with an empty matrix and less cristae since the beginning of treatment (9 h until to 48 h). After this time, the resistant cells show again a normal mitochondrial morphology while the sensitive cells are dying.

The elementary particles (F_1-ATPase) usually present on the negative stained cristae of isolated mitochondria, disappear from the sensitive cells while they are always present in the resistant cells (Curgy et al. 1980 a, b).

(3) Comparison between *Tetrahymena* and *Colpidium*

Results on morphology of mitochondria are more or less the same in *Colpidium* and in *Tetrahymena* (Curgy et al. 1980 a). But real sensitive cells of *Colpidium kleini* and *Colpidium campylum* are not found, even with 500 μg/ml. Cells grow more slowly than the resistant strain but they do not die. This fact can be related to the presence of bacteria in the medium, which can destroy or metabolize the CAP. This fact is rare: in the literature, only two species of trypanosomide were found to be insensible to CAP (250 μg/ml) (Eperon et al. 1983).

V. Cell Respiratory Capacity

A. Methods

— Cultures of a liter of medium with or without CAP (250 μg/ml) during 24 or 48 h, of *T. pyriformis*, *T. rostrata*, *T. vorax* and *C. kleini*

— O_2 consumption tests on concentrated cells.

Spectrophotometric study of respiratory enzymes on isolated and purified mitochondria.

— Cytochemistry with DAB (diaminobenzidine) for electron microscopy.

B. Results

(1) O_2 consumption decreases from 0 to 24 h in sensitive and resistant cells, continues to decrease in the sensitive cells but increases in the resistant cells from 24 to 48 h. This consumption is nearly the same as that of the control one (*Tetrahymena* species and *C. kleini*).

(2) After low temperature spectra of purified mitochondrial preparation, an important decrease of the concentration of cytochrome *b* and cytochrome-oxidase is observed in sensitive mitochondria treated during 24 or 48 h. This decrease is less important in resistant mitochondria (*Tetrahymena* species).

(3) The lack of cytochrome-oxidase in treated sensitive cells mitochondria is again corroborated by the lack of deposit on cristae after DAB reaction for electron microscopy (*T. pyriformis*).

These results confirm that some proteins as respiratory enzymes synthesized usually in mitochondria (in our *Tetrahymena* species studied, cytochrome *b* and a part of cytochrome-oxidase), are affected by CAP treatment. Cytochrome c is probably not synthesized in mitochondria in *Tetrahymena* because it is not affected (C u r g y et al. 1980 a, P e r a s s o et al. 1980 b, 1982).

VI. Modifications in Mitoribosomes

A. Methods

— Mass cultures (6 to 16 l according to strain and treatment) with CAP 250 µg/ml. Sensitive cells are inoculated 24 h before treatment to have enough ciliates for biochemistry.

— Electron microscopy: Karnovsky's method (1961); negative staining of isolated mitoribosomes.

— Biochemistry techniques:

— Protein extraction after mitoribosomes isolation

— Scintillation spectrometry

B. Results

(1) The electron microscopy with Karnovsky's method helps to show the significant decrease of mitoribosomes density in sensitive cell mitochondria (20%) and the important increase (40%) of them in resistant cell mitochondria after a CAP treatment of 24 and 48 h.

(2) The analysis of isolated mitoribosomes in sucrose gradient sedimentation, in electrophoresis on polyacrylamide gels and in negative staining by electron microscopy, shows the same result: CAP treatment breaks many mitoribosomes (80 S), principally of the sensitive cells, into their two subunits (55 S).

(3) Analysis of migration of mitoribosomal protein extracts in polyacrylamide gels after or not CAP treatment shows principally that two peaks lost in the sensitive treated cells are present in the resistant treated cells. Without treatment, only one other peak is absent in the resistant cells.

(4) The preferential fixation of CAP ^{14}C (100%) on the purified mitoribosomes of the sensitive cells, less strong (50 to 60%) on the purified mitoribosomes of the resistant cells, shows that the CAP is fixed "on" the mitoribosomes (Curgy et al. 1981, Perasso et al. 1982).

"The inhibition of the modified ribosomal progression on the messager RNA is the base of the molecular action of CAP on the protein synthesis" (Grivell et al. 1971).

VII. Proof of the DNA Mutation

A. Methods

— Cultures treated 24 h with CAP 250 µg/ml in 1 l Erlenmeyers,
— Mitochondrial DNA extraction with chloroform-phenol method,
— Action of restriction enzymes (Hind II, III, ECO RI),
— Electrophoresis in polyacrylamide gels.

B. Results

No deletion in the DNA fragments could be seen: the mutation is punctual, as in *Saccharomyces* and mammalian cells where one nucleotide was found responsible for the mutation (Dujon 1980, Blanc et al. 1981).

Conclusion

— CAP blocks the mitochondrial translational system of *T. pyriformis* by binding with mitoribosomes.

— In the mutant STR_1 the resistance character is due to a change in the mitoribosomes.

— This change consists in a loss or in a modification of one protein which is probably coded for by the mitochondrial genome.

— When the wild strain ST is grown in CAP containing medium:

(a) two bands are missing in the mitoribosomal protein electrophoresis pattern,

(b) the mitoribosome shows a tendency to subunit dissociation.

This tendency suggests that the mitoribosomal proteins translated into the mitochondria are involved in the assemblage of the two mitoribosomal subunits.

The others species studied show the same response to CAP treatment in their mitochondrial morphology and respiratory capacity.

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Action of a Heavy Ion, Cd^{2+}, and the Antagonistic Effect of Ca^{2+}, on Two Ciliates *Tetrahymena pyriformis* and *Euplotes vannus*

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Synopsis. The biochemical, ultrastructural and morphogenetic consequences of cadmium treatment were analyzed in two ciliates (*Tetrahymena* and *Euplotes*). Cadmium effects in *Tetrahymena* are suggested to result from a primary lesion at the transcriptional level. The morphogenetic effects observed in the two ciliates may result from Cd^{2+}/Ca^{2+} competition leading to cytoskeletal malfunctions.

Cd^{2+} ion is essentially a marine pollutant: its toxic effects on animal tissues and cell metabolism are now well known (Webb 1979). It has been tested on the marine dinoflagellate *Prorocentrum micans* by Prevot (1979) and Soyer and Prevot (1981). Mitochondria of this organism are modified by the Cd^{2+} treatment, like those of numerous organs in treated rat, as it was demonstrated in our laboratory (Toury et al. 1985). We compare some published results obtained in our laboratory from an axenic fresh water ciliate *Tetrahymena* (*Hymenostomatida*) (Pyne et al. 1983) with those obtained from a marine ciliate, *Euplotes vannus* (*Hypotrichida*) in monoxenic culture conditions (Curgy et al. 1982, Fleury et al. 1983) and we add unpublished biochemical data on *Tetrahymena*.

Material and Methods

A. Ciliate Culture

(1) *Tetrahymena pyriformis*

— Strain ST, amicronucleate (originating from Dr. Suyama's laboratory, Philadelphia, USA) was grown on P.P.Y.E. medium (proteose peptone 1.5%, yeast

extract 0.25%) and principally on defined medium from Rosenbaum et al. (1966) used with the high doses of Cd^{2+}.

— Strain GL, amicronucleate (from Cambridge culture collection) was grown on P. P. Y. E. medium only with low doses of Cd^{2+}.

(2) *Euplotes vannus* (from Fauré-Fremiet's ciliate culture collection). Five strains from different geographic origins were tested. *Euplotes* was grown in marine dried lettuce medium inoculated by *Enterobacter aerogenes*.

B. Cadmium Treatment

— A stock solution of $CdCl_2$ in distilled water is added after sterilization to the culture medium (time zero or 24 h) in order to obtain concentrations from 0.1 to 10 μg/ml for *Tetrahymena*. A dose of 20 μg/ml has been used to test the first reaction of *Euplotes*.

— In some experiments $CaCl_2$ was added with $CdCl_2$; the final concentration of $CaCl_2$ was four times that of the usual defined medium.

C. Methods

Specific techniques will be exposed at the beginning of each chapter.

I. Growth Pattern and Cell Morphology of *Tetrahymena*

Methods

— Cultures in 100 ml medium in Erlenmeyers with different added doses of Cd^{2+} and inoculated with about 1000 cells/ml.

Growth curves and generation time are deducted from cell counts.

— Cell morphology is controlled by:

— Protargol staining in optical microscopy,

— Usual fixations for electron microscopy (including Thiery's and Rambourg's (1974) and Bernhard's (1969) methods).

Results

(1) Growth Pattern and Optical Morphology:

— with low doses of Cd^{2+} (up to 1 μg/ml), *Tetrahymena* growth is not grossly affected. The generation time is longer but the swimming and the morphology of the cells are normal.

— with higher doses of Cd^{2+} the generation time is twice the normal one, or even more, and with 10 μg/ml in P.P.Y.E., almost all cells die. After several days, the surviving cells can grow; they become "adapted": Yamaguchi et al. (1978) showed that a "metallothionein like" protein was synthesized by cells and inactivated Cd^{2+}. In defined medium cells are protected, they don't die. In this case an excess of Ca^{2+} (three times the dose of the defined medium) has a more visible protective effect on cell growth. Without any Ca^{2+} ions in the defined medium,

cells die in four hours even in the presence of 5 µg/ml of Cd^{2+}. Other ions (Zn, Cu, Mg) do not seem to have such a visible protective effect.

— A dose of 10 µg/ml given to a 1000 cells/ml culture is worse than given to a 5000 cells/ml culture; in the latter case, the poison is less toxic because the dose is divided: there is a "population effect".

— A large number of cells treated with high doses have abnormal somatic and buccal ciliary systems, and furthermore an abnormal macronucleus, visible after protargol staining. After a 24 h treatment with 5 µg/ml Cd^{2+}, with or without Ca^{2+} in excess, 300 ciliates of each series have been counted after protargol staining, in order to determine the number of divisions. The percentage of dividing cells related to the generation time and length of the cell cycle is: 9% in control, 16% in presence of Cd^{2+}, and 14% in presence of Cd^{2+} + Ca^{2+}.

	Cell cycle	Duration of division
Control	5 h 30 min	30 min = 1/12th
Treated Cd^{2+}	20 h	3 h 12 min = 1/7th
Treated Cd^{2+} + Ca^{2+}	8 h	1 h 07 min = 1/8th

These results show that the lengthening of the total generation time results from a slowing down of the division process; after a few days in the culture, forms with abnormal kinetome and mouths evolve towards more normal or stable forms with 10% "doublets" cells (double kinetome and two opposite mouths) which can divide. With Ca^{2+} excess, doublets cannot arise, perhaps because ciliary disorders and macronuclear abnormalities are less frequent.

(2) Ultrastructural Morphology

— Mitochondria are not affected by Cd^{2+} treatment. But autophagic vacuoles, dense bodies, lipid granules increase highly. Endoplasmic reticulum forms whorls.

— Cd^{2+} treatment induces an important decrease of glycogen amounts (usually visualized with Thiery's method in normal cells cultivated with sucrose).

— Nuclear constituants are strongly affected by the first hour with 5 or 10 µg/ml: this indicates that nucleus is probably the first important target for cadmium toxicity; nucleoli aggregate and loose their granular aspect, their fibrillar part increases. Dense chromatin becomes larger and nucleoplasm clearer. These events remind us of the Actinomycin D action which inhibits transcription. They are more obvious after application of Bernhard's technique, with EDTA treatment. These morphological events are not modified by the presence of Ca^{2+} in excess (P y n e et al. 1983).

II. Decrease in Transcription

Methods

— Biochemical extraction of total RNAs after labelling with ^3H Uridine during the last hour before extraction, from control cells and cells treated with 10 μg/ml Cd^{2+} during four hours.

— Four hours migration in polyacrylamide SDS gels

— Migration in sucrose gradient (5-20%), followed by the radioactivity measure in a scintillation spectrometer.

— Radioautography in electron microscopy.

Results

The quantity of radioactivity measured on the purified RNA fractions after ^3H-U incorporation is very different between control and treated cells:

(1) the peak which corresponds to the precursor of rRNAs is increased ten times.

(2) the peaks corresponding to rRNAs 26 S and 17 S are decreased 2.2 times and 1.6 times respectively.

(3) the peaks corresponding to mRNAs, tRNA and 5 S RNA are decreased 1.3 time.

Results from electron microscopical autoradiography confirm the previous data. The radioactive molecule numbers in the macronucleus and in the cytoplasm of treated cells are 1.76 and 2 times less, respectively, than in control cells.

The following table summarizes these morphological and biochemical results:

		rRNAs		mRNAs, tRNAs, 5 S RNA	
		Fibrillar part of nucleolus	Granular part of nucleolus	Dispersed chromatin	Dense chromatin
Morphology	Control	++	++	++	++
	Treated cells	++++	+	+	++++
	Scintillation	precursor ×10	26 S:2.2 17 S:1.6	4 S and 5 S:1.3	
	Radioautography	nucleus: 1.76		cytoplasm: 2	

The Cd^{2+} effect on nuclear transcription is first the inhibition of the rRNAs maturation in the nucleolus: this induces the increase of its fibrillar part, suggesting that more precursors are conserved or retained. There are less mature rRNAs produced (26 S, 17 S): the granular

part of the nucleolus is obviously reduced. Nucleoplasm becomes clearer, this result means that the active chromatin is less abundant, and causes the mRNAs, tRNAs and 5 S RNA decrease.

III. Growth Pattern and Cell Morphology in *Euplotes*

Methods

— Test cultures in Petri dishes with 10 ml medium and 20 µg/ml Cd^{2+}

— Test cultures in depression slides with 1 ml medium and 10 µg/ml or 1 µg/ml Cd^{2+}, 10 cells being inoculated from an exponential phase culture stock.

— Growth curves are deducted from cells which have been hand counted everyday.

— Cell morphology is controlled by

— Protargol staining for optical microscopy

— Usual fixations for electron microscopy including Thiery's and Bernhard's methods.

Results

(1) Growth and optical morphology

— With 10 µg/ml or 1 µg/ml Cd^{2+}, the generation time resembles that of the control cells. But the stationary phase is reached with a reduced number of cells, which become black; four of the five tested strains die a few days after having been in stationary phase. Numerous abnormalities happened with the last strain: unusual selfing, physiological reorganization (Fleury and Fryd-Versavel 1981), gigantism associated with abortive divisions; monsters appear, which can include up to 30 recognizable attached cells. These last monsters can produce either:

— stable forms

(a) normal cells

(b) doublets or triplets forms which can reproduce when they are sustained

— or instable forms with excess of

(a) ventral ciliature
(b) dorsal ciliature

} which return more or less rapidly to the normal state.

A dynamic and comparative study of the various morphological states, obtained in the course of cadmium treatment, or heat shock in

Euplotes, shows that *Tetrahymena* and *Euplotes* give two similar responses to the Cd^{2+} agression, according to their own reaction capacity: vegetative reorganization in *Euplotes* and oral replacement in *Tetrahymena* are the first analogous reaction to chemical injury, the second one is the behaviour of the somatic cortex in *Tetrahymena* and dorsal face of *Euplotes* during cytokinesis. But the doublets do not have the same morphogenetical origin (Fleury et al. 1983).

(2) Ultrastructural morphology

— Mitochondria are not affected by Cd^{2+} treatment, but dense bodies, lipids and autophagic vacuoles increase. Lithosomes are greatly abnormal. The application of Thiery's method shows that:

— Cd^{2+} treatment induces an important decrease of the glycogen rosettes which are scattered in the cytoplasm.

— The polysaccharidic material usually accumulated in the secretory ampules associated with the ciliary system in this ciliate, lacks in these ampules and lies outside.

— The nuclear system is strongly affected, as in *Tetrahymena*: nucleoli change, chromatin spots become denser. The Bernhard's technique gives evidence of these modifications (Curgy et al. 1982).

Conclusion

The observations on *Euplotes*, and *Tetrahymena* treated with Cd^{2+}, indicate a nuclear transcription inhibition, which affects some secretory processes and leads to a slowing down and to transformations in the glucids and lipids metabolism.

This is in accordance with others authors' conclusions.

The morphogenetical disorder's origin is not well elucidated but is probably related to the competition between Ca^{2+} and Cd^{2+} ions. Indeed, we show here that Ca^{2+} ion presence greatly protects the cell morphology and physiology of *Tetrahymena*. Ca^{2+} ion is known for its importance at cytoskeleton level for positioning, attachment and movement of microtubules towards membrane. Cadmium is a good tool to investigate this problem.

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The Effect of Previous Brief Exposure to Colistin on *Tetrahymena pyriformis*

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Synopsis. The effects of brief (5 to 30 min) exposure of *Tetrahymena pyriformis* to the polypeptide antibiotic colistin were investigated. Earlier exposure to the drug was found to suppress the formation of a new oral apparatus and effect the resorption of oral primordia. Additionally, the ciliates exposed to colistin revealed a reduced phagocytosis rate. A complete recovery of all the investigated processes was observed. The findings seem to suggest that the duration of earlier exposure of the ciliates to colistin does not significantly affect the degree to which the physiological functions in question are disturbed.

The effects of cell metabolism inhibitors upon ciliates have been subject to numerous studies. Some species, e.g., *Tetrahymena* or *Chilodonella* were found to adapt to the continuous presence of low, nonlethal concentrations of the drug in the medium by means of physiological adaptation (Frankel 1965, 1969 b, 1970, Rasmussen and Zeuthen 1966, Satir 1967, Wunderlich and Peyk 1969, Nelsen 1970, Roberts and Orias 1974, Wang and Hooper 1978, Kiersnowska 1981). Disturbances in the physiological processes due to the addition of an inhibitor to the culture depended, on the one hand, on the specificity of the drug, its concentration and duration of the exposure, but also on the developmental stage and the general physiological status of the cell when exposed to the drug (Mitchison 1978).

The findings mentioned above concerned mostly inhibitors easily penetrating inside the cell, e.g., actinomycin (Nachtwey and Dickinson 1967, Satir 1967) or cyclohexemide (Frankel 1969 b, Heyer

and Frankel 1971, Kiersnowska 1981), and cell responses to both brief and continuous exposure to the drugs were investigated. The reports on ciliate reaction to exposure to agents which do not directly enter inside the cell are, however, scant (Németh and Csik 1961, Hayes 1977, Rebandel 1981, Rebandel and Karpińska 1981, Rebandel et al. 1981, Szablewski 1981, 1982), and deal essentially with response to the continuous presence of the inhibitor in the medium.

Earlier studies (Szablewski — in prep.), in which colistin was continuously present in the medium, demonstrated that *Tetrahymena* response to the agent was related to its concentration. Additionally, the ciliates adjusted to the employed antibiotic levels by means of physiological adaptation. The time of recovery depended then solely on the colistin concentration in the medium. A question remained to be answered, however, whether in case of an inhibitor which did not penetrate inside the cell the time of recovery would also depend on the duration of previous exposure of *Tetrahymena* to the antibiotic. The present study concerned the effects of short-time exposure to colistin upon the course of stomatogenesis and phagocytosis in *Tetrahymena pyriformis*.

Material and Methods

The ciliate used in the study was *Tetrahymena pyriformis*. Cultures were maintained axenically at 28°C in the medium containing 1.5% proteose-peptone + 0.1% yeast extract (Difco). Ciliates derived from the stationary phase were inoculated three times, every 24 h. Eighteen hours after the last inoculation the antibiotic was added to the culture at a concentration of 0.05 mM/l = 0.07 g/l. At various times thereafter (5 min, 10 min, 20 min and 30 min) the ciliates were washed out of colistin. *Tetrahymena* were washed three times with culture medium by centrifugation (5 min, 500 rotations/min). Next, the cells were transferred to flasks pre-warmed at 28°C and the culture medium was added up to 50 ml (Moment 0). Samples were removed at 0, 45 min, 120 min and 180 min. Specimens for the assay of stomatogenesis were prepared using a Chatton-Lwoff method, modified by Frankel and Heckmann (1968). Particular stages of formation of a new oral apparatus were determined according to Frankel (1969 a).

To assay phagocytosis, 1 ml of cell suspension was transferred to goblets at the appropriate time and 0.1 ml of ink suspension was added. After 15 min the cells were fixed with 0.1 ml 10% formalin solution. Subsequently the number of food vacuoles was counted in 100 cells.

The employed antibiotic colistin (polymyxin) was a mixture of colistin A and B (polymyxin E_1 and polymyxin E_2), produced by the Polfa Pharmaceutical Works at Tarchomin.

The results were compared using the "Two-way ANOVA with replication" variance analysis (Sokal and Rohlf 1969) at the confidence level of 95%.

Results

The Effect of Colistin on the Course of Stomatogenesis in *Tetrahymena pyriformis*

The study confirmed the effect of colistin upon the course of oral development in *Tetrahymena* (Fig. 1).

In the control sample the lowest fraction of cells with oral primordia was found at Moment 0 (5.4%). Their number subsequently increased to reach the maximum value 180 min after Moment 0, when cells with oral primordia constituted 14.8% of the control (Fig. 1).

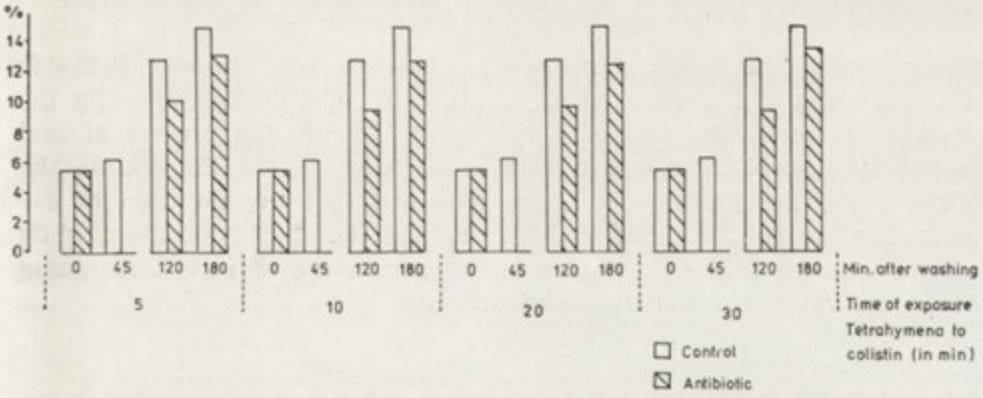

Fig. 1. The percentage of cells undergoing oral development, according to the duration of cell exposure to the antibiotic and the duration of the experiment

In colistin-treated cultures no ciliates undergoing stomatogenesis were found after 45 min, irrespective of the duration of previous exposure to the antibiotic. Cells with the developing oral apparatus were found after 120 min (9.3% to 10%), and 180 min after Moment 0 that fraction of cells rose to 13.3%, being essentially similar to the control level (Fig. 1).

The variance analysis revealed that the antibiotic addition produced a statistically significant inhibition of stomatogenesis as compared to control. On the other hand, however, it confirmed that a prolonged exposure of cells to colistin has not significantly affected the process.

A previous exposure of *Tetrahymena pyriformis* to the antibiotic also brought about a resorption of oral primordia in a number of ciliates. In the control cells no resorption of the oral apparatus could be observed in the course of the entire experiment. In the ciliates earlier exposed to the drug, however, a fraction of *Tetrahymena* undergoing

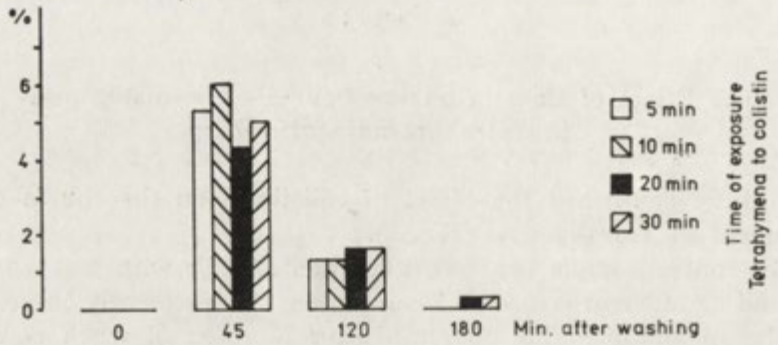

Fig. 2. The percentage of cells undergoing resorption of oral primordia, according to the duration of cell exposure to the antibiotic and of the experiment. No ciliates undergoing resorption of oral primordia were found in the control sample

resorption of the oral apparatus was observed after 45 min (4.3% to 6% according to the duration of exposure to colistin). Subsequently the number of such cells decreased and after 180 min the process of oral apparatus resorption was seen only in 0.3% of ciliates (Fig. 2). On the other hand, the variance analysis confirmed that the duration of exposure to the antibiotic had no effect on the course of resorption, while the differences between samples in the percentage of cells undergoing resorption were not statistically significant.

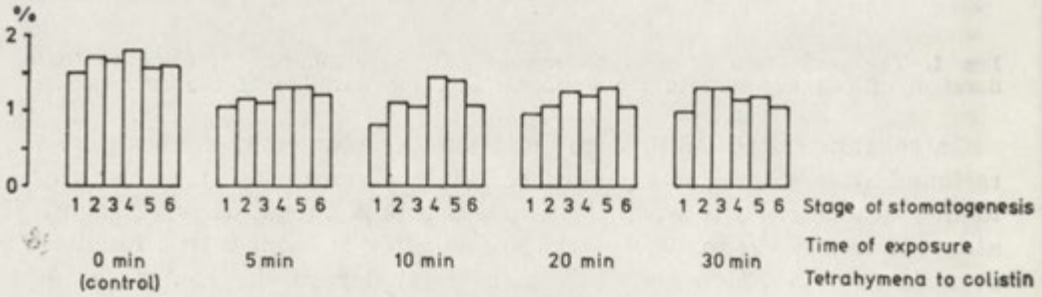

Fig. 3. The percentage of cells undergoing particular stages of oral development, according to the duration of cell exposure to the antibiotic. The percentage is given by the formula:

$$\% \text{ cells} = \frac{\% \text{ cells in a particular stage of stomatogenesis}}{\text{number of samples}}$$

The analysis of the effect of colistin upon the distribution of frequency of particular stages of oral development in *Tetrahymena* revealed no significant deviation from the distribution found in controls (Fig. 3). The observed differences in the distribution of particular stages of stomatogenesis between experimental cultures and control and among samples treated with colistin were not statistically significant.

The Effect of Colistin upon the Process of Phagocytosis in *Tetrahymena pyriformis*

The present experiment was concerned merely with the rate of endocytosis and did not deal with the process of egestion.

As in the case of oral development, colistin was found to affect the process of phagocytosis in *Tetrahymena pyriformis*. In the control sample the average number of food vacuoles in a cell was 7.29. After the addition of antibiotic the number of food vacuoles decreased in all the investigated samples. Subsequently the number gradually increased to reach the control value at different times, according to the duration of ciliates exposure to colistin (Table 1). In cells exposed to the drug for 5 min, 10 min and 20 min, recovery occurred within 45 min, while with exposure lasting 30 min the control level was achieved after 120 min (Table 1).

Table 1

The mean number of food vacuoles in a cell and the standard deviation according to the duration of cell exposure to the antibiotic and the duration of the experiment. In control the values are 7.29± ±0.15, respectively

Time of exposure of *Tetrahymena* to colistin	Min. after washing			
	0	45	120	180
5 min	6.41±0.40*	7.38±0.26	7.22±0.38	7.10±0.49
10 min	6.59±0.46*	6.95±0.21	7.20±0.42	7.08±0.47
20 min	6.16±0.58*	6.89±0.40	7.28±0.68	7.25±0.14
30 min	6.49±0.36*	6.62±0.17*	7.00±0.21	7.20±0.16

* statistically significant decrease in the number of food vacuoles in a cell as compared to control, (for Tables 1 and 2).

The results obtained by other authors (Nilsson 1979) indicate that in *Tetrahymena* food vacuoles develop in succession, one after another. The average time of food vacuole formation calculated for the control sample was 2.05 min. Following the addition of the antibiotic at Moment 0, the time of food vacuole formation increased for all experiments (Table 2) and the difference observed between control and colistin-treated cultures was statistically significant. On the other hand, the variance analysis confirmed that the precise increase in the time of food vacuole formation was not related to the duration of previous exposure of cells to the drug. The distribution of the percentage of cells with a given number of food vacuoles as affected by the culture conditions was studied as well (Table 3). A previous exposure of *Tetrahymena* to colistin was not found to cause any shift in the cells to adapt to the

Table 2

The mean time of food vacuole formation (min) and the standard deviation as affected by colistin. In control the value is 2.05 min

Time of exposure of *Tetrahymena* to colistin	Min. after washing			
	0	45	120	180
5 min	2.34±0.10*	2.03±0.06	2.07±0.26	2.11±0.15
10 min	2.27±0.13*	2.15±0.05	2.08±0.13	2.12±0.40
20 min	2.43±0.26*	2.17±0.11	2.06±0.20	2.07±0.05
30 min	2.31±0.11*	2.26±0.02*	2.14±0.05*	2.08±0.03

continuous presence of the drug in the medium is referred to as the physiological adaptation (Nyberg et al. 1978). The acclimation, or adaptation has been suggested to involve the formation in the cell of still unknown macromolecules responsible for the alteration in the permeability to the given inhibitor in its passage into and/out of the cell (Frankel 1970, Roberts and Orias 1974).

Two problems investigated in the present study require a more thorough analysis.

(1) With inhibitors of cell metabolism penetrating inside the cell, the relevant factors include the duration of cell exposure to the inhibitor (Mitchison 1978). However, the response of *Tetrahymena* to colistin, which does not enter inside the cell, seems to depend mostly on the concentration of the drug in the medium.

(2) Why is the time of recovery in the process of stomatogenesis different from that observed in the process of phagocytosis?

The first phenomenon might be accounted for by different rates at which inhibitors penetrate inside the cell, lower in case of inhibitors entering inside the cell than in the event when colistin is employed. The results obtained might also suggest a two-stage reaction of colistin with the components of cell membrane.

The other problem is more difficult to explain. The process of phagocytosis returns to normal more rapidly than the process of oral development, although both involve the cell membrane. The suppression of phagocytosis in *Tetrahymena* or a decrease in its rate due to an addition of colistin to the culture medium lasts until the cell membrane within the cytostome has been fully recovered, structurally as well as functionally. Therefore, there are no aftereffects brought about by the inhibitor. On the other hand, secondary effects of colistin should be considered in case of distribution of cells with a given number of food vacuoles. The differences observed between control and experimentals were not statistically significant.

Table 3

The mean percentage of cells with a given number of food vacuoles as affected by colistin. The percentage is given by the formula:

$$\text{mean \% cells} = \frac{\text{\%cells with a given number of food vacuoles}}{\text{number of samples}}$$

	Number of food vacuoles in cell															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	3.25	0.40	1.60	2.21	3.66	7.06	10.52	15.07	22.92	15.66	14.31	2.07	1.07	0.00	0.20	0.00
Antibiotic	4.66	1.28	1.93	2.47	4.75	7.54	10.90	16.41	20.66	14.55	11.55	2.33	0.72	0.07	0.11	0.07

Discussion

The results obtained demonstrate that a fairly short exposure of ciliates to colistin (5 min) is sufficient to inhibit the course of the investigated physiological functions of the cells. The findings by other authors have been similar. Newton (1955) investigating the effect of polymyxin B (as sulphate salt) upon *Pseudomonas aeruginosa* showed that a 5 min exposure of the bacteria to the drug led to its building into the cell structures. According to the literature, the polymyxins, including colistin, do not enter inside the cell, but are incorporated into the cell membrane through a combination with lipopolysaccharides (Kuryłowicz 1979). The results obtained seem to suggest that the antibiotic becomes incorporated into the cell membrane fairly rapidly. Also, since the effects brought about by an addition of colistin to the culture tend to persist (over 120 min in some cases), they may be considered either a permanent combination or a secondary disorder of physiological processes due to colistin.

Our findings revealed that after some time the investigated physiological functions returned to normal, following the initial blockage effected by an addition of colistin to the medium, thus demonstrating the capacity of *Tetrahymena pyriformis* to resume normal physiological functions. However, the mechanism of such recovery remains unknown. Studies on the continuous presence of colistin in the culture medium of *Tetrahymena pyriformis* (Szablewski — in prep.) established the time of recovery from the effects of the antibiotic similar to the one observed in cases of short-time exposures to the same concentrations of the drug. The capacity of stomatogenesis, particularly affecting the resorption of oral primordia. In *Tetrahymena* the resorption is an "all-or-none" process and once started, it must go to completion (Frankel 1967 a). Accordingly, although a given cell metabolism inhibitor has ceased to exercise its effect upon the cell, the resorption of the oral primordium must continue. The process is also known to be related to the phenomenon of a set-back in the cell cycle of *Tetrahymena* (Frankel et al. 1980). Before the process of stomatogenesis is started, the cell cycle must return to the earlier stage and only then the new oral apparatus may develop. Additionally, it might be also necessary to consider the role of turnover, since according to Frankel (1967 b) the resorption of oral primordia is likely to be related to the process.

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Action of Calcium Blockers on Potassium-induced Reversed Beat of Cirri in *Stylonychia mytilus*

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Synopsis. Three inorganic calcium blockers (Co^{2+}, Mn^{2+}, Ni^{2+}) and two organic ones (Verapamil, D-600) showed in *Stylonychia mytilus* a marked effect on the duration of reversed beat of cirri which was associated with activation of voltage sensitive calcium channels within ciliary membrane. In ciliates exposed to solution 20 mM KCl + 1 mM $CaCl_2$ + 1 mM Tris/HCl (pH 7.2)a complete inhibition of potassium-induced reversed beat of cirri could be observed in case of Co^{2+} and Mn^{2+} action whereas in case of Ni^{2+}, Verapamil and D-600 even at sublethal contractions only decrease of duration of cirri beat reversal was noticed. The achieved results suggest that *Stylonychia* may serve as a very useful tool in studies on effects of calcium blockers at the cellular level.

Extensive studies on excitability and behaviour of ciliate protozoa brought evidence that both fresh water (Kinosita et al. 1964, Dryl 1970, 1974) and marine ciliates (Dryl et al. 1982) may respond to various external stimuli with short or longer lasting depolarization of the cell membrane associated with ciliary reversal. It was shown more recently that reversed beat of cilia is due to influx of free external calcium ions throughout voltage sensitive Ca-channels located within ciliary cell membrane (Eckert 1972, Naitoh 1974). It should be pointed out that *Stylonychia mytilus* shows also above mentioned electrophysiological and behavioural responses towards external stimulation (Machemer 1971, Machemer and de Peyer 1977). *Stylonychia* possess several groups of compound cilia (so-called cirri) which react in analogous way as single cilia. At appropriate concentration of external calcium ions ($\geqslant 10^{-6}$M) the reversed beat of cirri (CR) induced

by external stimuli is associated with activation (opening) of hypothetical voltage sensitive calcium channels in similar way as in other ciliates and in excitable cells of metazoa (Fatt and Ginsborg 1959, Hagiwara et al. 1969). So the induction and duration of CR response caused by potassium ions in *Stylonychia* can be treated as an indicator of activation of calcium channels within cell membrane of compound cilia.

The aim of the present study is to analyze the possible effect of some well known inorganic and organic Ca-blockers on the excitability of *Stylonychia mytilus* exposed to higher concentrations of KCl. The authors wished also to compare the results with experimental data known from studies on metazoan excitable cells.

Material and Methods

Experiments were carried out on *Stylonychia mytilus* strain (isolated from small pond in Warsaw surroundings) cultivated in Pringsheim medium with addition of *Tetrahymena pyriformis* as a source of food. Ciliates were kept at room temperature 20-22°C, 6 h before starting experiment being washed three times in solution: 1 mM $CaCl_2$ + 1 mM Tris (HCl) pH 7.2. KCl and Ca-blocker substances were also prepared on the basis of above mentioned solution.

It was proved during preliminary series of experiments that 20 mM KCl is not toxic to *Stylonychia* and induces CR lasting approximately 120 s. This behavioral effect was easy to measure since the end of backward movement of *Stylonychia* along circles is more or less abruptly marked by slow swimming forwards or complete immobilization. The criterion for finished CR response was observation that 50% of ciliates do not show more CR. The average duration (in seconds) of CR was calculated on the basis of ten observations in samples containing ca. 20-25 specimens.

In experimental series the ciliates were preincubated for 3 min in solution containing tested Ca-blocker substance before adding KCl in desired concentration.

In order to obtain the reliable results it appeared necessary to determine the toxic effects of investigated Ca-blockers. Care was taken that the highest applied concentration should not exceed the dose LD_{50} (during 1 h of exposure) in solution 20 mM KCl containing the Ca-blocker substance.

Results and Discussion

Toxic effects of Ca-blockers could be established on the following levels: LD_{50} (1 h exp.) for tested substances were — 6.4×10^{-3} M $CoCl_2$, 6.4×10^{-3} M $MnCl_2$, 3.2×10^{-4} M $NiCl_2$, 5×10^{-6} M Verapamil and 5×10^{-6} M D-600.

Inorganic blockers (Co^{2+}, $MnCl_2$) proved to be approximately 1000 times less toxic than organic blockers Verapamil and D-600. $NiCl_2$ was more toxic than remaining inorganic Ca-blockers and at all applied concentrations induced slowering of movement due to discoordination of beat in various groups of cirri.

In higher concentrations of $CoCl_2$ and $MnCl_2$ the movement of ciliates after 2 min of exposure was slowed down and during that time disappeared spontaneous short lasting CR responses and CR responses towards mechanical stimulation. The ciliates were swimming slowly forwards along right-winded arcs instead of normally observed movement along left-winded arcs (Fig. 1). The direct observations under low po-

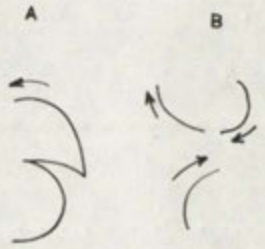

Fig. 1. Movement of *Stylonychia mytilus* in medium devoid of Ca-blockers (A) and after 2 min of incubation in medium containing Ca-blockers in sublethal concentrations (B). Observation from dorsal side of swimming ciliates

wer optical microscope brought evidence that the observed disturbance of movement is caused by inhibiting effects of applied cations on the motile activity of right marginal cirri. Verapamil and D-600 caused also after 2 min of exposure slackening of the forward movement and decrease in number of spontaneous CR responses.

The ciliates preincubated for 3 min in solutions containing Ca-blockers and afterwards exposed to 20 mM KCl solution — showed gradual decrease of CR duration parallel with application of higher concentrations of tested substances. As indicated on Fig. 2 in case of all five Ca-blockers the registered values of CR duration could be arranged along lines of different steepness. Complete inhibition of CR was noticed only in highest applied concentrations of $CoCl_2$ and $MnCl_2$ while in the case of $NiCl_2$ Verapamil and D-600 CR response could not be abolished even at concentration corresponding to LD_{50} (1 h exp.).

It should be emphasized that duration of K^+-induced CR was analyzed by Kamada and Kinosita (1940) in their classical study on effects of external potassium and calcium ions on behaviour of *Paramecium*. On the basis of their data Jahn (1962) has calculated that duration of K^+-induced CR depends on ratio $[K^+] : [Ca^{2+}]^{1/2}$ and his

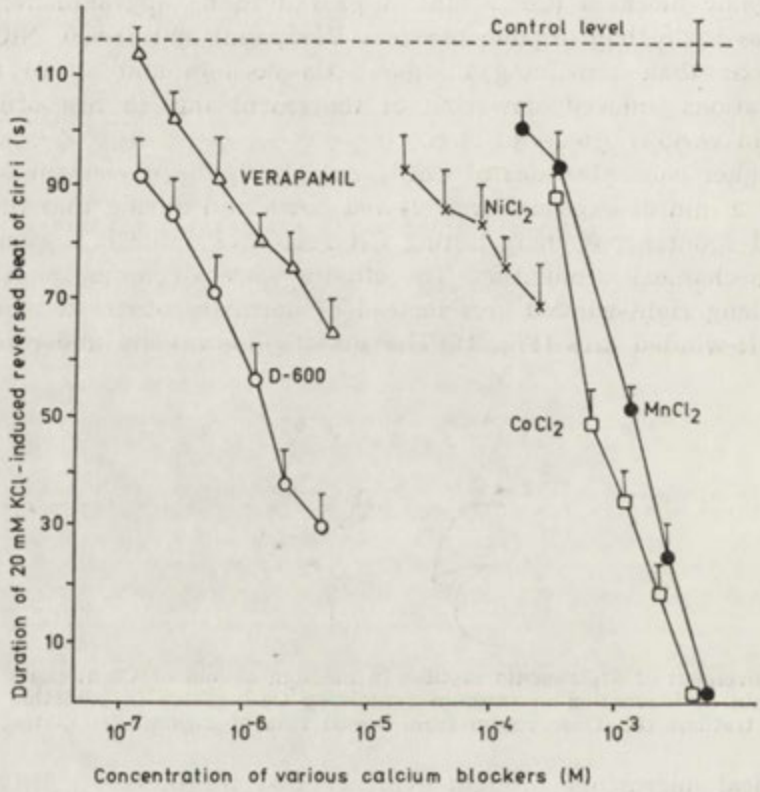

Fig. 2. Suppresion of K^+-induced reversed beat of cirri (CR) in *Stylonychia mytilus* by increasing concentrations of Ca-blockers. The dose-response curves are close to straight lines of different steepness. The points represent average values from ten measurements with corresponding SE

view was supported by Grębecki (1964, 1965) who indicated that the observed changes of behaviour in *Paramecium* may depend on degree of decalcification or recalcification of the cell membrane by action of potassium or other cations added to external medium. The idea of a competitive action of external cations on the cell membrane bound calcium is generally accepted (Naitoh 1968, Dryl and Jahn 1974) and found further confirmation in more recent studies (Hildebrand and Dryl 1976). In *Stylonychia mytilus* the threshold for induced CR by potassium ions was also proved to depend on Gibbs-Donnau's ratio (Dryl and de Peyer 1970). In view of the above mentioned findings the decrease of CR duration by Ca-blockers can be also explained by their possible action on the cell membrane bound calcium. Although the detailed explanation of observed efects of Ca-blockers is not yet possible — it should be noted that very similar cations competitive action

of Ca-blockers was suggested by Langer (1973) in case of excitation-contraction coupling in heart muscle.

It is interesting that concentrations of Ca-blocker solutions applied in this study are analogous to those causing loss of contractility in cardiac muscles of some vertebrates (Kaufmann and Fleckenstein 1965, Fleckenstein 1983). This may suggest rather surprising functional similarities of calcium sensitive channels in unicellular *Stylonychia* and in metazoan cells. However, unlike in metazoan cells, in *Stylonychia* the organic Ca-blockers (Verapamil and D-600) proved to be less potent than inorganic ones.

Strong inhibiting action of Co^{2+} and Mn^{2+} on excitability of *Stylonychia* is also evident from observed abolition of spontaneous short lasting CR responses which are associated with occurrence of depolarizing action potentials (Machemer 1977).

Another interesting effect of Ca-blockers was a marked decrease of swimming rate of *Stylonychia* and suppression of motile activity of marginal cirri already after 2 min of incubation in solution with higher concentration of Ca-blockers. This phenomenon cannot be explained by blocking calcium voltage sensitive channels located within ciliary membrane. The mechanism of above mentioned reactions is obscure, since it is not known how the external factors may influence the motile activity of cilia or cirri.

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The Factors Affecting the Cultivation of the Rumen Ciliate Protozoon *Entodinium exiguum in vitro*

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Synopsis. The rumen ciliate *Entodinium exiguum* was anaerobically cultured in four different culture media. The ciliates did not survived when a salt solution corresponding to ruminant saliva was used as a culture medium and when the food consisted either of hay alone or of hay and pure barley starch. Death of the ciliates also occurred when the medium preferred by these organisms was supplemented with βsitosterol. The food consisting of ground hay (30 per cent), barley flour (47 per cent) and wheat gluten (23 per cent) allowed the protozoa to survive for 6 months with a population doubling time varying from below 24 h to over 94 h. Addition of soluble carbohydrates to the food caused a decrease of the population density of *Entodinium exiguum*. Supplementation of the culture medium with choline chloride, I-cysteine or soluble protein did not affect the concentration of ciliates in the cultures.

During the recent years it has been shown that the presence of ciliates in the rumen decreases microbial N synthesis (Demeyer and Van Nevel 1979) and adversely affects the liveweight gain and wool growth of the host (Bird and Leng 1978, Bird et al. 1979). These results might neccessitate a revision of the common belief that the rumen protozoa are symbiotic microorganisms and point out the need of a better knowledge of their biology. *In vitro* studies seem to be very useful here since they make possible the maintenance of single-species

This study was carried out partially under the project coordinated by the Institute of Animal Physiology and Nutrition, Jabłonna near Warsaw.

cultures of the rumen ciliates. Hitherto only few species commonly occurring in the rumen have been extensively studied *in vitro* (Sugden 1953, Gutierrez 1959, Coleman 1960, 1969 a, Coleman et al. 1972, 1976, 1977, Mah 1964). Our observations have shown that *Entodinium exiguum* is also a common constituent of the rumen microfauna. The results of a study on the maintenance of this species outside its natural environment are presented in this paper.

Materials and Methods

The protozoa used in this experiment were taken from the rumen of sheep. The initial culture was obtained by inoculation of 1 ml rumen fluid into Erlenmeyer flasks containing 39 ml of medium and food. The food consisted of equal amounts of hay and barley flour. The daily ration was 40 mg per culture. The cultivation was performed by the method routinely used in our laboratory. The ciliates were fed every day and every fourth day they were transferred into a fresh medium according to Michałowski (1975 b). During the precultivation period some species of protozoa died. The cultures in which *Entodinium exiguum* was the only surviving species were used for inoculation of experimental cultures.

Four types of salt solution were used for cultivation of the ciliates (Table 1). Choline chloride (Serva) in the quantity 0.25 μg/ml/day; βsitosterol (Sigma Chem. Co.) in the quantities 0.05 or 0.025 mg/ml/day and L-cysteine (Reanal) in the quantity 0.2 mg/ml every fourth day were added to the salt solution. Standard solutions

Table 1

The chemical composition of salt solutions used for cultivation of *Entodinium exiguum* (g/l)

Ingredient	A	B	C	D
K_2HPO_4	6.3	4.9	0.0	0.0
KH_2PO_4	5.0	3.8	1.0	0.0
$NaH_2PO_4 \times 12H_2O$	0.0	0.0	0.0	9.3
$NaHCO_3$	0.0	6.6	5.0	9.8
KCl	0.0	0.0	0.0	0.57
NaCl	0.65	0.49	6.0	0.47
$CaCl_2 \times 6H_2O$	0.09	0.07	0.20	0.08
$MgCl_2$	0.0	0.0	0.0	0.06
$MgSO_4 \times 7H_2O$	0.09	0.07	0.2	0.0
CH_3COONa	0.75	0.0	0.0	0.0
pH	6.84	7.53	7.76	8.52

A — "caudatum type" salt solution, B — "simplex type" salt solution (both according to Coleman et al. 1972). C — "Hungate type" salt solution (according to Hungate 1942). D — artificial saliva (after McDougall 1948).

of the substances mentioned were prepared according to Coleman (1962) and Broad and Dawson (1976).

Ground hay, barley flour, pure barley starch, pectins, cellulose (Koch Light Lab. Ltd.), glucose, galactose, xylose, raffinose, sucrose (Polskie Odczynniki Chemiczne), wheat gluten, phaseolin, bovine serum albumin (Down Development and Chemicals Ltd.) and casein (BDH Ltd.) were used for food preparation. The basic food ration was 40 mg/culture flask/day. Pure barley starch was obtained by the method of Whelan (1955) and wheat gluten and phaseolin according to Klein (1933) and Pace (1955). The solubility of the proteins used in the "*caudatum* type" salt solution was 15, 57, 100 and 100 per cent for wheat gluten, phaseolin, serum albumin and casein, respectively.

The cultivation began by inoculation of 20 ml of *Entodinium exiguum* suspension from the precultivation flasks into flasks containing 20 ml of culture salt solution and food. The cultivation procedure was the same as described above (Michałowski 1975 b). Three cultures were always run for each factor studied.

The samples for protozoa counts were taken every fourth day and fixed in equal volume of 4 per cent formalin solution. The number of protozoa in the samples were estimated under light microscope using the method of Michałowski (1975 a). Each sample was counted three times. The obtained results were statistically analysed using Student's *t*-test.

Results

The number of *Entodinium exiguum* in relation to the medium used is presented in Fig. 1. The average population density on "simplex type" medium was 10.8×10^3/ml while that on "Hungate type" salt solution 12.2×10^3/ml. The "caudatum type" salt solution made possible the maintenance of 34×10^3/ml cells of *Entodinium exiguum* ($P < 0.05$).

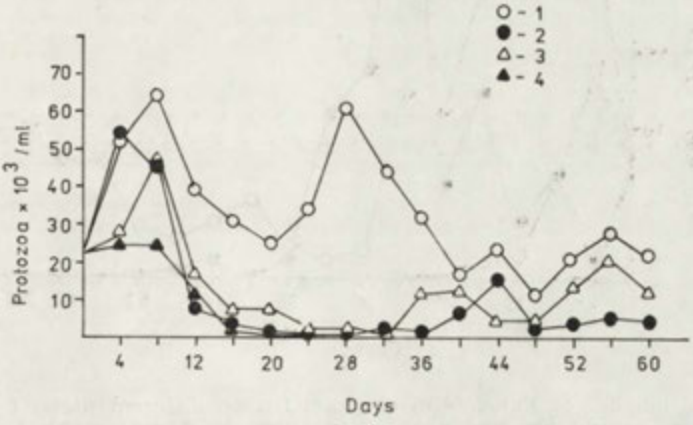

Fig. 1. The number of *Entodinium exiguum* cultivated in "*caudatum* type" salt solution (1), "simplex type" salt solution (2), "Hungate type" salt solution (3) and artificial saliva (4)

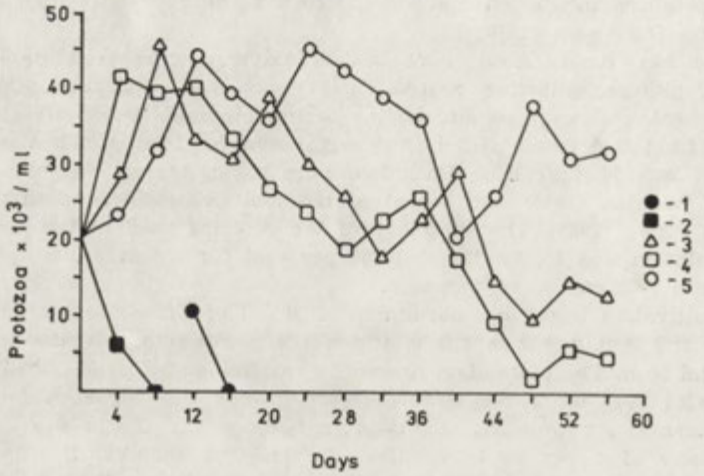

Fig. 2. Effect of supplementation of "*caudatum* type" salt solution with βsitosterol at 0.025 mg/ml/day (1) or 0.05 mg/ml/day (2), with choline at 0.25 μg/ml/day (3), with L-cysteine at 0.2 mg/ml every fourth day (4), control cultures (5). All the cultures were fed on 40 mg of food containing of ground hay (30 per cent), barley flour (47 per cent) and wheat gluten (23 per cent)

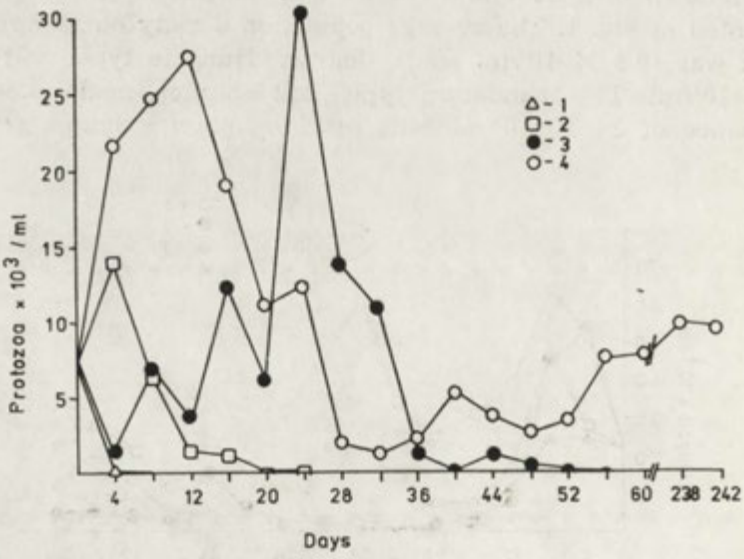

Fig. 3. The number of *Entodinium exiguum* fed on different diets: *1* — hay, *2* — a mixture of ground hay and pure barley starch (50 per cent each), *3* — a mixture of barley flour (70 per cent), and wheat gluten (30 per cent), *4* — a mixture of ground hay (30 per cent), barley flour (47 per cent) and wheat gluten (23 per cent). The daily ration was 40 mg/culture flask

The use of McDougall artificial saliva caused a steady decrease of the number of protozoa. All the cultures were fed on a mixture containing ground hay (30 per cent), barley flour (47 per cent) and wheat gluten (23 per cent).

The addition of 1 or 2 mg βsitosterol/culture flask/day to the "*caudatum* type" culture medium caused a rapid fall in the number of ciliates (Fig. 2). The addition of choline or L-cysteine had no effect on population density as compared to the control cultures ($P > 0.05$).

When the cultures were fed on hay alone the ciliates survived only for 8 days (Fig. 3). The supplementation of hay with pure barley starch prolonged the survival of protozoa up to 20 days, and the food containing barley flour (70 per cent) and wheat gluten (30 per cent) up to 60 days. The protozoa survived over 240 days on the mixture of ground hay (30 per cent), barley flour (47 per cent) and wheat gluten (23 per cent). The doubling time of the population in these cultures measured over 60 consecutive days varied from below 24 to over 94 h.

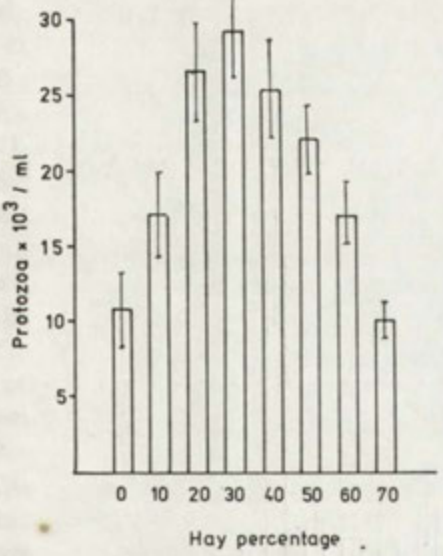

Fig. 4. Mean number of *Entodinium exiguum* in the cultures fed on mixture of different proportions of ground hay, barley flour and wheat gluten. The daily ration of food was 40 mg/culture. The content of barley flour was 70, 62, 55, 47, 39, 31, 23 and 16 per cent respectively and that of wheat gluten — 30, 28, 25, 23, 21, 19, 16 and 14 per cent

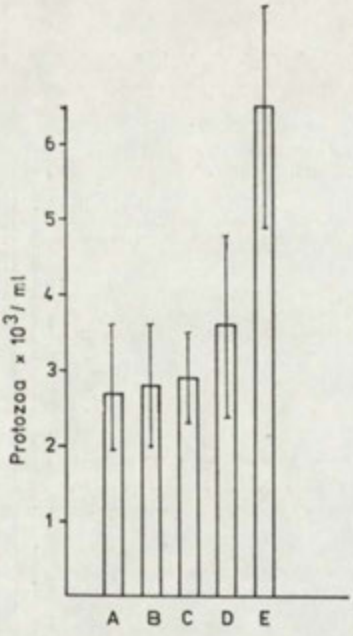

Fig. 5. The mean number of *Entodinium exiguum* in the cultures on different proteins. The control diet (*A*) had hay (12 mg) and barley flour (19 mg) and was supplemented with (*B*) bovine serum albumin, (*C*) casein, (*D*) phaseolin and (*E*) wheat gluten (9 mg each)

The number of *Entodinium exiguum* in the cultures fed on food containing ground hay, barley flour and wheat gluten is shown in Fig. 4. The cultivation period lasted 40 days. The number of the ciliates was the highest in the cultures fed on 30 per cent hay, 47 per cent barley flour and 23 per cent wheat gluten, and did not differ from those recorded in the cultures receiving 20, 40 and 50 per cent hay in the food ($P > 0{,}05$). The concentration of protozoa in the cultures receiving 0, 10, 60 and 70 per cent of hay was less ($P < 0.01$).

The influence of protein solubility on the number of *Entodinium exiguum* is shown in Fig. 5. The protozoa were fed on a mixture of ground hay, barley flour and protein in the proportion of 30, 47 and 23 per cent, respectively. The control diet contained only hay and barley flour but no additional protein. The experiment lasted 64 days. The ciliate number in the cultures fed on soluble protein was the same as in the control cultures. The population density of *Entodinium exiguum*

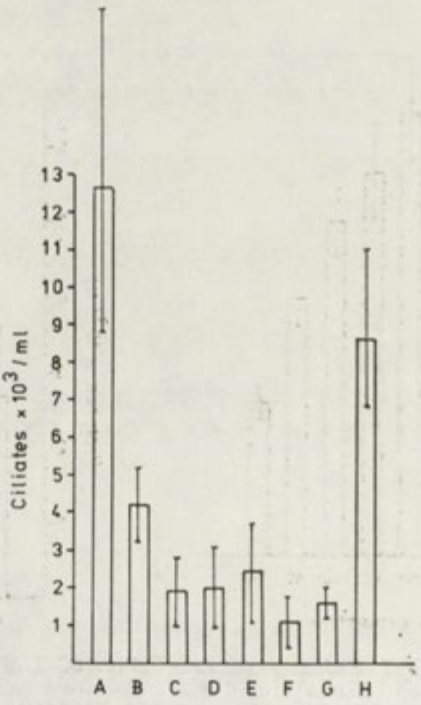

Fig. 6. Effect of different carbohydrates on the population density of *Entodinium exiguum*. The control diet (40 mg/culture) consisted of ground hay (30 per cent), barley flur (47 per cent) and wheat gluten (23 per cent) and was supplemented with 5 mg of : glucose (*B*), galactose (*C*), raffinose (*D*), xylose (*E*), sucrose (*F*), pectin (*G*), cellulose (*H*). A — control cultures with no carbohydrate supplement

increased with decreasing protein solubility and was the highest (6.4×10^3/ml) on wheat gluten.

The supplementation of food with soluble sugars or with pectin reduced the number of ciliates from 12.6×10^3/ml to 1.2×10^3/ml in the cultures with 5 mg sucrose added (Fig. 6). There were no significant differences in the concentration of protozoa between the control cultures and those supplemented with cellulose ($P > 0.05$).

Discussion

Of all the four culture media used, the "*caudatum* type" salt solution gave the best growth of *Entodinium exiguum in vitro*. This medium seems to be preferred also by other species of rumen ciliates (Coleman et al. 1972, 1976, 1977). On the contrary, the artificial saliva (McDougall 1948) caused a rapid death of protozoa which might be a consequence of a relatively high pH value of this medium (Table 1). The pH of "*caudatum* type" salt solution is about 6.8 and seems be optimum for rumen ciliates (Michałowski 1975 b). On the other hand, the decrease of pH was probably the cause of protozoa death in the cultures supplemented with βsitosterol.

Broad and Dawson (1976) showed that choline was required by *Entodinium caudatum* for growth and the positive influence of choline became visible on food with no choline. Cysteine might affect the rumen ciliate by changing the red-ox potential of the medium. In our experiment neither choline nor cysteine increased the number of *Entodinium exiguum*. The relatively rich diet (hay, barley flour and wheat gluten) might have caused the uneffectiveness of both these supplements.

The ciliates died rapidly when the food consisted only of hay. On the other hand, the lack of hay in the diet limited their number. The presence of hay (or dried grass) in the food seems to be necessary for the long-term maintenance of *Entodinium exiguum in vitro*.

The concentration of *Entodinium exiguum* increased when protein solubility decreased. A similar relationship have been observed in *Entodinium caudatum* (see the next paper). It seems plausible that both these ciliates have a limited capability of taking up soluble protein. It can not be excluded that under such conditions the ciliates engulf more bacteria which grow on excess of soluble protein.

The rumen ciliates of the family *Ophryoscolecidae* have a limited ability to utilize soluble carbohydrates (Abou Akkada and Howard 1960, Wiliams et al. 1961, Coleman 1969 b, 1972). The

excess of these nutrients may then cause a rapid growth of bacteria and, as consequence of bacteria fermentation — a decrease of pH. Such changes in the environment of ciliates might have a detrimental effect on the growth of protozoa in cultures supplemented with readily soluble carbohydrates (Fig. 6).

The ciliates survived *in vitro* for may months although the doubling time of the population was scarcely shorter than 24 h. The slow growth of Entodinium exiguum may be due to the *in vitro* conditions different from those in the rumen and also to our incomplete knowledge of the nutritive and environmental requirements of this organisms. Further investigation are therefore necessary.

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The Influence of Protein on the Number of Rumen Ciliates *Entodinium caudatum* and *Diploplastron affine in vitro*

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Synopsis. The rumen ciliates *Entodinium caudatum* were cultured separately or together with *Diploplastron affine*. Ground hay mixed with either barley flour or with pure barley starch and supplemented with different levels of protein was used as food, and salt solution composed of (g/l) : K_2HPO_4 — 6.3, KH_2PO_4 — 5.0, $MgSO_4 \times 7H_2O$ — 0.09, NaCl — 0.65, $CaCl_2 \times 6H_2O$ — 0.09, CH_3COONa — 0.75 (Coleman et al. 1972) as a culture medium.

The ciliates died when the food consisted of hay and barley starch even when supplemented with purified protein up to 10 per cent of daily ration. The survival of protozoa was observed either when the food consisted of hay and barley flour (containing 9.3 per cent of protein) or when the food consisting of hay and barley starch was supplemented with purified protein not less than 15 per cent of daily ration.

The addition of 15 per cent of wheat gluten to the food consisting of hay and barley flour increased the population density of *Entodinium caudatum* from 2.6×10^3/ml to 7.2×10^3/ml.

Wheat gluten and hordein caused the greatest increase in *Entodinium caudatum* number and vicilin gave the best development of *Diploplastron affine* among all the 16 proteins tested.

The population density of ciliates was negatively correlated to protein solubility. The negative correlation between bacteria and *Diploplastron* number on food supplemented with globulins was also observed. The bacteria density about 10^7/ml had no prolongation effect on the ciliates survival *in vitro*.

Many studies relating to the factors affecting the rumen ciliate population have been performed both *in vivo* and *in vitro*. The factors studied were: the diet (Abe et al. 1973, Michałowski 1975 a, b) and feeding frequency (Moir and Somers 1956, Michałowski

and Muszyński 1978). These experiments showed that both the increase in feeding frequency and the supplementation of food with starch-rich constituents affected favourably the ciliate population.

Much less attention was paid to the influence of the dietary protein. It was studied *in vivo* by Mowry and Becker as early as 1930. More recently the *in vitro* studies were performed by Einszporn (1961) and Bonhomme-Florentin (1974). The scarcity of the data related to this problem is probably due to the fact that rumen ciliates engulf large quantities of bacteria which were recognized as a major (if not the sole) protein source for ciliates (Coleman 1972, Coleman and Laurie 1974), and thus the effect of the dietary protein could be masked by the bacteria ingestion.

The doubts about the role of dietary protein for the ciliate nutrition made us carry out studies on the significance of this protein for the development of the ciliate population.

Materials and Methods

The experiments were carried on the populations of *Entodinium caudatum* and *Diplodinium affine* from the rumen of sheep fed on hay and concentrates. Rumen fluid samples of about 100 ml were taken 4-6 h after feeding and treated as described elsewhere (Michałowski 1975 b). Each experiment was preceded by a pre-cultivation period when the ciliates were fed on hay and barley flour. Within this period some of the ciliate species disappeared and those that remained were *Entodinium caudatum*, *Diplodinium affine* and often *Entodinium exiguum*. The cultures with established protozoal fauna were then used for experiments.

Experiment 1. The influence of protein supplementation on the *Entodinium caudatum* number was investigated. The experimental culture were fed on 40 mg of the mixture consisting of 12 mg hay, 22 mg barley flour and 6 mg wheat gluten. The protein content in hay and barley flour was 9.0 and 9.3 per cent D. M. respectively. The control cultures received 34 mg mixture of 12 mg hay and 22 mg barley flour., Three cultures were fed on each kind of food. The cultivation period lasted 64 days.

Experiment 2. The effect of the protein content in the diet on the population density of *Entodinium caudatum* and *Diplodinium affine* was studied. The food (50 mg/culture/day) consisted of hay, pure barley starch and soya protein. The content of hay was either 30 or 50 per cent. The control cultures received the food without any protein supplementation, while the experimental food was supplemented with soya protein up to 5, 10, 15, 20, 25 and 30 per cent. Six cultures were maintained simultaneously on each food. The experiment lasted 40 days.

Experiment 3. The number of *Entodinium caudatum* and *Diplodinium affine* in relation to the kind of protein supplemented was studied. The food (50 mg/culture flask/day) consisted of hay (30 per cent), pure barley starch (55 per

cent) and protein (15 per cent). Sixteen proteins were tested, among which 3 were albumins, 6 — globulins and the remaining 7 formed the third group called "other proteins". The amino-acid content of the protein used is presented in Tables 1-3, while the protein solubility in "*caudatum* type" salt solution — in Table 6. Six cultures were fed on each protein. The experiment was performed in three 20 day periods devoted to albumins, globulins and "other proteins".

The experimental cultures were initiated by inserting 20 ml inoculum to the 50 ml Erlenmeyer flask with 20 ml of the culture medium and food. The "*caudatum* type" salt solution (Coleman et al. 1972) was used as the culture medium. The solution consisted of (g/l) : K_2HPO_4 — 6.3, KH_2PO_4 — 5.0, NaCl — 0.65, $CaCl_2 \times 6H_2O$ — 0.09, $MgSO_4 \times 7H_2O$ — 0.09, CH_3COONa — 0.75. The cultivation was performed by the method described earlier (Michałowski 1975 b). Every day the cultures were fed and then saturated with a stream of CO_2. Every fourth day half of the culture volume was removed after precise mixing and 20 ml of fresh medium added. Then the cultures were fed.

The 2 ml samples for counting the microorganisms were taken from the discharged part of the culture every fourth day and fixed with 2 ml of 4 per cent formalin solution. The ciliates were counted as described by Michałowski (1975 a). The bacteria number was determined using Thoma counting chamber. Each sample was counted three times.

The mean number of microorganisms with its standard deviation was calculated from the obtained results. The differences between mean values were analysed using the analysis of variance. The correlation coefficient between the protein solubility and the protozoa number as well as between bacteria and pro-

Table 1

The amino acid content of albumin used for food supplementation (μmol amino acid/culture/day)

Amino acids	Chicken egg albumin	Bovine serum albumin	Leucosin
Asp.	5.76	5.10	1,67
Thr.	2.88	3.06	1.37
Ser.	6.48	2.45	2.42
Glu.	9.36	6.88	9.33
Pro.	2.67	2.34	4.56
Gly.	3.42	1.43	2.21
Ala.	4.50	3.64	2.66
Val.	5.04	2.86	2.22
Met.	2.88	0.20	0.26
Ile.	4.49	1.07	1.41
Leu.	5.76	5.13	3.10
Tyr.	1.62	1.55	1.11
Phen.	3.78	2.32	1.38
His.	1.26	1.49	0.56
Lys.	3.60	4.67	0.79
Arg.	2.70	1.84	1.47
Try.	0.54	0.22	0.41

Table 2

The amino acid content of globulins used for food supplementation (μmol amino acid/culture/day)

Amino acids	Glycinin	Vicilin	Phaseolin	Legumin	Conglutinin	Tuberin
Asp.	6.78	8,07	5.89	5.97	6.59	2.49
Thr.	1.92	1.78	1.73	1.89	1.60	1.45
Ser.	4.38	4.39	3.41	3.18	4.18	3.19
Glu.	8,65	10.55	9.47	8.21	18.08	9.86
Pro.	2.56	3.18	3.25	3.03	2.75	4.77
Gly.	3.13	3.18	3.17	3.58	3.40	0.81
Ala.	2.66	2.66	2.43	3.23	2.04	6.32
Val.	3.39	3.26	2.79	3.12	2.06	1.81
Met.	0.22	0.12	0.18	0.15	0.61	0.32
Ile.	2.58	2.95	2.20	2.15	2.31	1.80
Leu.	4.86	5.97	3.81	4.27	5.47	9.50
Tyr.	1.92	1.33	1.20	1.32	1.12	1.62
Phe.	3.39	2.93	2.13	2.10	1.89	2.48
His.	1.98	1.15	1.00	1.18	1.35	0.43
Lys.	2.99	6.15	2.23	1.93	1.79	1.51
Arg.	1.80	2.66	2.33	3.34	4.89	0.52
Try.	0.48	0.35	1.15	0.45	0.38	1.21

Table 3

The amino acid content of "other proteins" used for food supplementation (μmol amino acid/culture/day)

Amino acid	Zein	Hordein	Gliadin	Gluten	Glutenin	Casein	Gelatin
Asp.	7.67	0.70	1.50	1.71	1.96	3.62	3.74
Thr.	1.93	0.76	1.48	1.51	1.83	2.38	1.44
Ser.	2.14	1.84	3.43	3.06	2.73	3.04	2.75
Glu.	4.71	17.40	18.80	16.41	10.64	10.25	6.01
Pro.	1.68	12.14	8.37	6.41	4.07	5.85	8.19
Gly.	2.90	0.57	1.97	2.91	3.59	1.95	19.75
Ala.	2.19	0.57	2.45	1.85	2.04	2.61	8.84
Val.	2.25	1.95	2.57	1.99	1.76	3.70	1.93
Met.	0.25	0.11	0.43	0.31	0.14	0.49	0.21
Ile.	1.56	1.47	2.22	1.63	1.52	2.52	0.87
Leu.	3.12	2.63	4.28	3.17	3.37	5.16	2.11
Tyr.	1.19	0.98	1.04	1.10	1.31	2.09	0.31
Phe.	1.51	3.44	2.45	1.96	1.53	2.23	1.17
His.	0.45	0.41	0.89	0.87	0.87	0.54	0.29
Lys.	2.13	0.18	0.46	0.66	1.06	3.08	1.91
Arg.	1.72	0.60	1.20	1.19	1.46	1.46	4.64
Try.	0.04	0.59	0.42	0.35	0.99	0.50	0.00

tozoa number was also calculated. The average densities of microorganisms in individual cultures fed on certain proteins were used for correlation coefficient calculations (six cultures were received each kind of protein). The calculations were performed according to Bailey (1966).

The majority of proteins were obtained in our laboratory. The commercial products were only: chicken egg albumin (Koch Light Laboratories Ltd.), bovine serum albumin (Down Development and Chemicals Ltd.), casein (BDH Ltd.) and gelatin (Merck). The proteins were obtained by the methods described by Klein (1933) and Pace (1955). Glycinin was purified from the commercial soya protein; tuberin was obtained from potato bulbs, while other proteins from the seeds of respective plant. The pure barley starch was obtained by the method of Whelan (1955).

The protein solubility was determined as follows: the precisely weighed portions of protein were suspended in 10 ml of "*caudatum* type" salt solution, mixed throughly for 5 min and allowed to stay overnight. The suspension was then centrifuged (10 min, 1.000 r.p.m.) and the protein content in the supernatant was estimated by Folin Phenol Reagent. Each determination was performed three times. The amino acid content was estimated after 16 hours' hydrolysis of protein in 6 NHCl at 107°C in anoxygenic conditions. The amino acid content was then determined using Durrum automatic amino acid analyser. The tryptophan content was determined by xanthydron method (Greenstein and Winitz 1961).

Results

The population density of *Entodinium caudatum* in the cultures fed on ground hay, barley flour and wheat gluten was between 2 × 10^3/ml and 14 × 10^3/ml, while there was no more than 5 × 10^3/ml ciliates in the control cultures (Fig. 1). The mean number was 2.6 × 10^3/ml and 7.2 × 10^3/ml in the control and experimental cultures respectively. The

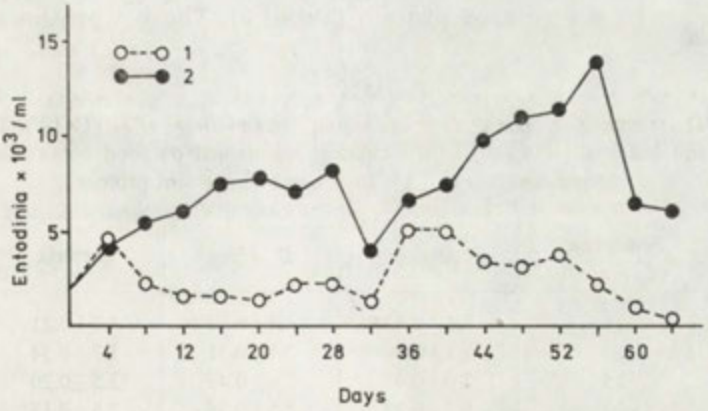

Fig. 1. The effect of food supplementation with wheat gluten on the population density of *Entodinium caudatum*. *1* — The cultures fed on hay and barley flour. *2* — The cultures fed on hay, barley flour and wheat gluten

difference (4.6 × 10^3/ml) is statistically significant at one per cent probability.

In cultures fed on 30 per cent of hay and 70 per cent barley starch, as well as in those where protein content was 5 per cent, the protozoa died within 8 days and when the food contained 10 per cent protein, the ciliates survived no longer than 20 days. The repeated cultivation gave similar results. In the cultures fed on food containing 15, 20, 25 and 30 per cent of protein the protozoa survived till the end of cultivation period — 40 days. Their mean concentration in relation to protein content in food is presented in Table 4. The day to day variations

Table 4

The number of *Entodinium caudatum* and *Diploplastron affine* (×10^3/ml) in the cultures maintained on food containing 30 per cent of hay and 15, 20, 25 and 30 per cent protein

Percentage of protein	*Entodinium caudatum*	*Diploplastron affine*
15	1.2±0.31*	0.2±0.07
20	0.9±0.17	0.2±0.02
25	1.4±0.50	0.2±0.05
30	1.1±0.22	0.1±0.05

* Mean value ± S.D.

in the ciliate number were observed. They were the smallest in the cultures receiving food with 15 per cent of protein. In the second variant of this experiment, food containing 50 per cent of hay was offered. The highest number of *Entodinium caudatum* was noted in the cultures fed on food with 15 per cent of protein (Table 5). There were no significant

Table 5

The number of *Entodinium caudatum*, *Diploplastron affine* (×10^3/ml) and bacteria (×10^7/ml) in the cultures maintained on food containing 50 per cent hay and 15, 20, 25 and 30 per cent protein

Percentage of protein	*E. caudatum*	*D. affine*	Bacteria
15	3.6±0.37*	1.4±0.17	3.7±0.23
20	2.5±0.29	2.3±0.31	3.7±0.34
25	2.0±0.47	2.6±0.49	3.5±0.20
30	1.5±0.29	2.5±0.34	3.6±0.17

* Mean value ± S.D.

differences in the number of bacteria and in the number of *Diploplastron affine* in the cultures receiving 20, 25 and 30 per cent of protein in the food.

The ciliate number in the cultures fed on different proteins varied from 2 × 10^2 to 52 × 10^2/ml and from 0.1 × 10^2 to 5 × 10^2/ml for *Entodinium caudatum* and *Diploplastron affine* respectively (Table 6).

Table 6

The number of *Entodinium caudatum*, *Diploplastron affine* (×10^2/ml) and bacteria (×10^7/ml) in the cultures fed on different proteins

Proteins	Solubility of proteins	*E. caudatum*	*D. affine*	Bacteria
chicken egg albumin	100	14.0±1.99*	0.7±0.04	5.4±0.43
(1) bovine serum albumin	100	13.1±1.88	0.3±0.18	5.9±0.47
leucosin	100	12.0±3.47	0.5±0.18	5.2±0.58
glicinin	35	14.1±5.55	4.0±0.40	3.5±0.29
vicilin	48	6.0±1.10	5.0±0.90	4.1±0.49
(2) phaseolin	57	3.2±0.45	4.0±1.88	4.7±0.42
legumin	78	5.0±1.55	4.0±1.18	4.3±0.29
conglutinin	100	2.0±0.45	1.0±0.62	6.1±0.83
tuberin	100	12.9±0.09	0.1±0.04	3.5±0.43
zein	3	50.1±4.03	0.9±0.02	2.6±0.40
hordein	3	44.9±3.65	0.2±0.05	3.7±0.80
gliadin	12	43.0±4.65	0.2±0.07	4.3±0.45
(3) gluten	15	52.0±2.93	0.3±0.16	3.1±0.29
glutenin	17	45.9±7.77	0.1±0.05	3.1±0.25
casein	78	16.1±2.22	0.2±0.05	3.5±0.38
gelatin	84	16.9±2.46	0.1±0.02	3.3±0.40

* Mean value ± S.D., (1) — albumins, (2) globulins, (3) — "other proteins". Solubility of proteins in per cent.

The lowest number of *Entodinium caudatum* was observed in cultures fed on globulins and the *Diploplastron affine* number was highest. Inversely, in the cultures fed on "other proteins" the concentration of *Entodinium caudatum* was highest, while only 10-100 cells of *Diploplastron affine* per ml of cultures were visible. A negative correlation was found between the solubility of globulins and the concentration of *Entodinium caudatum* and *Diploplastron affine*. Similarly, the correlation between the solubility of "other proteins" and the concentration of *Entodinium caudatum* was also negative (Table 7). A negative correlation was also found between the number of bacteria and *Diploplastron affine* fed on globulins.

Table 7

The probability of correlation between protein solubility and ciliate number as well as between bacteria and ciliates number

Correlated parameters	Correlation coefficient	Degrees of freedom	Statistical significance at the level of
Globulin solubility vs. *Ent. caudatum*	−0.658	34	0.001
Globulin solubility vs. *Dipl. affine*	−0.483	34	0.01
"Other protein" solubility vs. *Ent. caudatum*	−0.813	40	0.001
Bacteria vs. *Ent. caudatum* on globulins	−0.056	28	n. s.
Bacteria vs. *Dipl. affine* on globulins	−0.396	28	0.05
Bacteria vs. *Ent. caudatum* on "other proteins"	−0.225	28	n. s.

Discussion

The addition of protein to the diet consisting of hay and barley flour substantially increased the population density of *Entodinium caudatum*. Previously Einszporn (1961) showed that the increase in the dose of wheat gluten or gliadin enlarged the number of *Entodinium longinucleatum*. Both these results indicated that the ciliate number might be related to the amount of offered dietary protein.

The maintenance of living protozoa in cultures receiving hay and pure barley starch needed the supplementation of at least 15 per cent protein. It suggested that hay and bacteria alone could not cover the protein requirement of ciliates. The reason of this fact may be the low accessibility of hay protein for ciliates on the one hand, and a low number of bacteria on the other. The concentration of bacteria in the rumen is above 10^{10}/ml (Bryant and Robinson 1961) while the highest concentration of these organisms in our experiments was 6.5×10^7/ml (Table 6). It seems possible that in such conditions the ciliates were unable to engulf enough number of bacteria to cover their protein requirement. This suggestion is supported by the observations on the relation between the density of bacteria population and their number engulfed by rumen protozoa (Coleman 1964, 1972, Coleman and Laurie 1974).

The comparison of the results of Experiment 3 (Table 6) with the data presented in Tables 1-3 does not suggest any relation between the ciliate population density and the content of particular amino-acids in the proteins offered. On the contrary, the effect of protein solubility on the protozoa number is well documented (see Table 7 and Fig. 2).

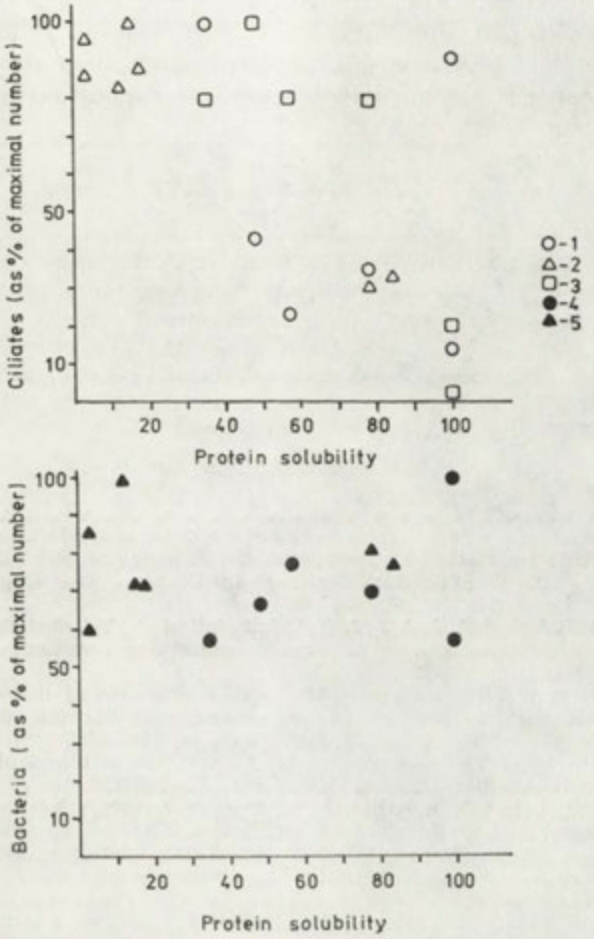

Fig. 2. The influence of dietary protein solubility on the population density of rumen ciliates and bacteria. The population density on different proteins is expressed as the percentage of the highest microorganism number obtained on particular groups of proteins (see Table 6). *1* — *Entodinium caudatum* on globulins, *2* — *Entodinium caudatum* on "other proteins", *3* — *Diploplastron affine* on globulins, *4* — bacteria on globulins, *5* — bacteria on "other proteins"

The low ciliate number in cultures maintained on soluble proteins may be the consequence of the fact that these organisms are probably unable to ingest the nutrients dissolved in the medium (H u n g a t e 1955). Although the more recent investigations (C o l e m a n 1967, 1969) suggest that e.g., amino acids and glucose penetrate the ciliate cells, but the mechanism of their uptake is quite different than the mechanism of ingestion of such a high-molecular compound as protein. If it is like that, the increased contribution of soluble protein to the diet will de-

crease the protein pool available for the ciliates; hence worse conditions for their growth and therefore better bacterial development. Perhaps the ciliates, being in such conditions, can balance their protein deficiency by more intensive ingestion of bacteria. This suggestion needs, however, experimental support.

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Cytochemical Demonstration of Some Hydrolytic Enzymes in Some Gregarines (*Protozoa* : *Sporozoa*). I. The Nonspecific Acid and Alkaline Phosphatases

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Synopsis. Non-specific acid and alkaline phosphatases have been demonstrated cytochemically in the gregarine *Stylocephalus conoides*. In addition, the former enzyme was also demonstrated in other four gregarine species, *S. mesomorphi*, *Gregarina cuneata*, *Hirmocystis speculitermis* and *Didymophyes minuta*. In the light of our findings, probable functions of these enzymes are suggested.

In spite of a number of papers reported on phosphatase activity in some protozoans, both parasitic and free-living, it is evident that the activities of these enzymes in gregarines have not been studied so far. The present paper reports cytochemical findings of non-specific acid phosphatase at different stages of the life-cycle of a gregarine *Stylocephalus conoides* and in trophozoites of *Gregarina cuneata*, *S. mesomorphi*, *Hirmocystis speculitermis* and *Didymophyes minuta* (*Protozoa*, *Sporozoa*). Non-specific alkaline phosphatase was also observed in trophozoites of *S. conoides*.

Materials and Methods

Trophozoites of *Stylocephalus conoides* taken out from the fore-gut of its host, a beetle *Opatrum* sp. and squash, preparations of gametocysts, gametes, spores and sporozoites were frozen at —50°C over dry ice vapours for 15-20 min and thawed before using them for cytochemical investigations. Similarly, trophozoites of *S. mesomorphi*, *Gregarina cuneata*, *Hirmocystis speculitermis* and *Didymophyes minuta* were obtained from their respective hosts: beetle *Mesomorphus velliger*, larvae of *Tenebrio molitor*, termites *Speculitermes cylops sinhalensis* and larvae of the beetle *Scarabeus* sp.

For demonstration of non-specific acid phosphatase the Naphthal AS-phosphate azo dye method of Burston (1962) (see Pearse 1968) was followed. The above mentioned stages of gregarines were incubated for 2-3 h in the medium containing AS-TR phosphate as a substrate, a diazonium salt Fast red TR, 0.2 M acetate buffer (pH 5.3) and a few drops of 10% $MnCl_2$ as an activator. Trophozoites incubated in the substrate-free medium served as controls.

In the trophozoites of *S. conoides* two other aspects of this enzyme activity were also studied:

(1) The effect of pH variation of the incubating medium;

(2) The effect of host starvation on the enzyme activity in the parasites.

The details of the procedures are as follows:

Variation of pH Values of the Substrate Medium

Groups of five trophozoites were separately incubated in the media identical in all respects except the pH. The pH values of these media were 5.9, 5.6, 5.3, 5.0, 4.5 and 2.9 respectively. After the routine processing the enzyme activities were assessed by visual gradations.

Effect of Host Starvation on the Parasite's Enzyme Activity

The pH values of the freshly collected beetle's fore-gut fluid contents was checked up with pH papers. The gregarine trophozoites from the corresponding samples of the hosts were incubated in the above mentioned standard substrate medium. Similarly, pH values were recorded for the fore-gut fluid samples of the hosts-beetles subjected to starvation for respectively 24, 36, 48, 60, 72 and 96 h. Trophozoites obtained from the respective host samples were incubated separately, and the intensities of enzyme activities were assessed by visual gradations.

For the study of non-specific alkaline phosphatase the Naphthol AS-phosphate azo dye method of Burston (1962) (vide Pearse 1968) was followed. The incubation medium contained AS-TR phosphate as a substrate, fast red TR as a diazonium salt, 0.2 M "tris" hydroxyl aminomethane buffer at pH 9.5 and 0.005 mg $MnCl_2$ as an activator. Trophozoites were incubated for 1 h during which time the incubation medium was renewed twice. Samples of trophozoites incubated in the substrate-free medium served as controls.

Observations

Non-specific Acid Phosphatase Activity

The non-specific acid phosphatase activity was observed in the form of AS-TR deposits in the trophozoites of *S. conoides, S. mesomorphi, G. cuneata, H. speculitermis* and *D. minuta* (Pl. I 1-5). The enzyme

was observed both in the ectoplasm and endoplasm of trophozoites, though a little more in the former. The control samples did not show any type of deposits (Pl. I 6). The activity was very feeble in the zygotes and spores as evidenced by very few AS-TR deposites (Pl. I 7).

Effect of pH Variation in the Incubation Medium on the Acid Phosphatase Activity in *S. conoides*

Trophozoites incubated in the medium with pH 5.9 showed a very feeble activity, while those incubated in the medium with pH 5.6 and 5.3 displayed a considerable enzyme activity (Pl. I 1). Another drop in the enzyme activity again was followed in the media with pH values equal to 5.0 and 4.5. The enzyme activity was in traces and confined only to the endoplasm in specimens incubated in the medium with the pH 2.9 (Pl. II 8).

Effect of Host Starvation on Acid Phosphatase Activity in *S. conoides* Incubated in the Substrate Medium with pH 5.3

Trophozoites from the hosts starved for 1-36 h showed a weak enzyme activity and pH values of the fore-gut fluid from where these parasites were procured, ranging between 6.5 and 6.0. The hosts that starved for 48 h contained a smaller amount of nutrient fluid in the fore-gut, pH values of this fluid, ranging between 5.5 and 5.0. The enzyme activity of trophozoites from this sample of hosts well compared with that in the trophozoites from non-starved hosts. The fore-gut of the host beetles that starved for 60 h contained very little fluid material and was full of gas bubbles. The pH value of this fluid was ranging between 4.5 and 4.0. The parasites from such hosts showed a very weak enzyme activity. After 72 h of starvation the fore-guts of the hosts were shrunken; no gas bubbles or fluid material were in traces and pH value was near 3.0. The parasites obtained from such hosts showed only traces of the enzyme activity (Pl. II 9). The hosts that starved for 96 h contained only dead trophozoites.

Non-specific Alkaline Phosphatase Activity

The activity of non-specific alkaline phosphatase was observed in the form of brownish black deposits in the ectoplasm and endoplasm of the trophozoites of *S. conoides* (Pl. II 10 and 11). Fine brownish black granules were also found scattered in the epimerite too. Control specimens showed no granules at all.

Discussion

Non-specific acid phosphatase activity has been demonstrated in several protozoa other than gregarines by cytochemical methods (Carrera and Changus 1948, Balamuth 1950, Blumenthal et al. 1955, Sen Gupta and Ray 1955, Hunter 1957, 1959 a, 1961, 1963, Elmofty 1957, Brins 1960, Beyer 1961, Beyer et al. 1977 a, b, 1978, Muller et al. 1962, Klamer and Fennel 1963, Lavette 1959, Sergejeva 1964, Frandsen 1968, Esteve 1970, Ohashi 1971 and Amoji 1975). In the present work we have observed the enzyme activity in the trophozoites of the gregarines *Stylocephalus conoides, S. mesomorphi, Gregarina cuneata, Hirmocystis speculitermis* and *Didymophyes minuta*. Feeble activity has also been observed in the zygotes and spores of *S. conoides*. However, the sporozoites of this species showed no enzyme.

The pH optima for acid phosphatase activity are different for different protozoa. These values are 4.5 in *Entamoeba histolytica* (Blumenthal et al. 1955) and *Amoeba proteus* (Muller et al. 1962) and 5.2 in *Nyctotherus georgei, Opalina ranarum* and *O. coracoides* (Amoji 1975). And that our present studies on gregarine species showed values ranging between 5.6 and 5.3. At any shift in the pH value on either side of this range, the enzyme activity is retarded. This point is amply supported by our findings on the acute drop in the intensity of this enzyme activity in the parasites examined from the hosts that starved for more than 48 h. Further, it is interesting to observe a mild enzyme activity in the parasites from the unstarved hosts. Since the pH value of the ambient medium ranges between 6.5 and 6.0, it is natural in this case to notice rather a feeble activity of the enzyme.

Localization of non-specific acid phosphatase has been reported for a few protozoans. It was noticed conspicuously around the food vacuoles in *Chaos chaos* (Brins 1960), around the nucleus and phagosomes in *Trichomonas vaginalis* and *T. tenax* (Ohashi 1971), in the parabasal apparatus in *Trichonympha turkestanica* (Elmofty 1957), in *Spirotrichonympha* sp. (Lavette 1959), and around the food vacuoles and buccal membranes in *Nyctotherus cordiformis* (Sergejeva 1964). In the gregarine species under report an intense activity was observed in the epimerite of *Stylocephalus conoides* and *S. mesomorphi*, and in the basal region of the protomerite of *D. minuta*. In addition to this enzyme, the epimerite of *S. conoides* also shows two lytic enzymes, i.e., hyaluronidase and β-glucuronidase (unpublished data). This enables us to ascribe a lytic role to the epimerite. It might be envisaged that the

embodded portion of the epimerite brings about lysis of the host gut epithelium by releasing the aforesaid enzymes that helps in drawing nourishment from the macerated tissue.

Cyclicity in the acid phosphatase activity has been studied in two protozoans. In *Tetrahymena pyriformis* a low activity was observed in fresh cultures, and an increased activity was followed in the cultures of 12 days or more, in organisms with an active glucose-uptake, however, there was a drop in the enzyme activity (Klamer and Fennel 1963). In *Nyctotherus cordiformis*, increasing activity of the enzyme during the trophic growth has been noticed along with a steady accumulation of glycogen, the results were negative in the winter-autumn forms which had accumulated glycogen to the maximum, acid phosphatase was feeble in the cysts (Sergejeva 1964). Our observations on the activity of this enzyme in the life cycle of *S. conoides* are almost identical with those of Sergejeva (1964). Therefore it seems reasonable to correlate this activity in protozoa with the uptake of nutrient materials — carbohydrates and lipids — during their vegetative growth.

The occurrence of non-specific alkaline phosphatase has been reported in a number of protozoa other than gregarines (Gill and Ray 1954, Sen Gupta and Ray 1955, Beyer 1961, Beyer et al. 1977 a, b, 1978, Das Gupta 1961, Ray and Hajra 1962, Haldar and Chakravarty 1963, Sharma and Bourne 1964 b, c, Frandsen 1968, and Perez-Reyes and Streber 1968). The present communication is the first report on the activity of non-specific alkaline phosphatase in trophozoites of the gregarine *S. conoides*.

Opinions regarding the functions of the non-specific alkaline phosphatase vary. Gill and Ray (1954) suggest a functional correlation between the high concentration of RNA and an intense activity of this enzyme in the karyosomes during protein synthesis and also in the regulation of membrane permeability in *Eimeria tenella*. Using tritiated glucose MacMillan (1973) has shown that the epimerite of *Nematocystis magna* facilitates the intake of metabolites from the host's tissue. Ormiere's (1977) ultrastructural studies on *Pyxinia firmus* corroborate MacMillan's views. Our present studies on the enzyme activities in the epimerite of *S. conoides* show that this body part has several hydrolytic enzymes including non-specific alkaline phosphatase. It would not be unreasonable to visualise a role played by this enzyme in regulating the permeability of metabolites taken up from the macerated host's tissue by the epimerite of the parasite. However, this point needs confirmation.

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[1] Part of Ph. D. Thesis accepted by Karnatak University, Dharwad, India.

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EXPLANATION OF PLATES I-II

Photos 1-9 — Non-specific acid phosphatase activity in gregarine trophozoites. 10 and 11 — Non-specific alkaline phosphatase activity in *S. conoides*

1: *Stylocephalus conoides.* Anterior portion of a trophozoite, × 100

2: *S. mesomorphi,* Anterior portion of a trophozoite, × 400

3: *Gregarina cuneata.* A young trophozoite, × 100

4: *Didymophyes minuta.* A syzygy of trophozoites, × 100
Note an intense enzyme activity in the basal region (arrow) of the primite's protomerite

5: *Hirmocystis speculitermis.* A magnified portion of the deutomerite of a trophozoite showing the enzyme activity in the ectoplasm, × 1000

6: *Stylocephalus conoides.* Anterior portion of a trophozoite incubated in the substrate-free medium, × 200. A control preparation. Note the near absence of the enzyme activity

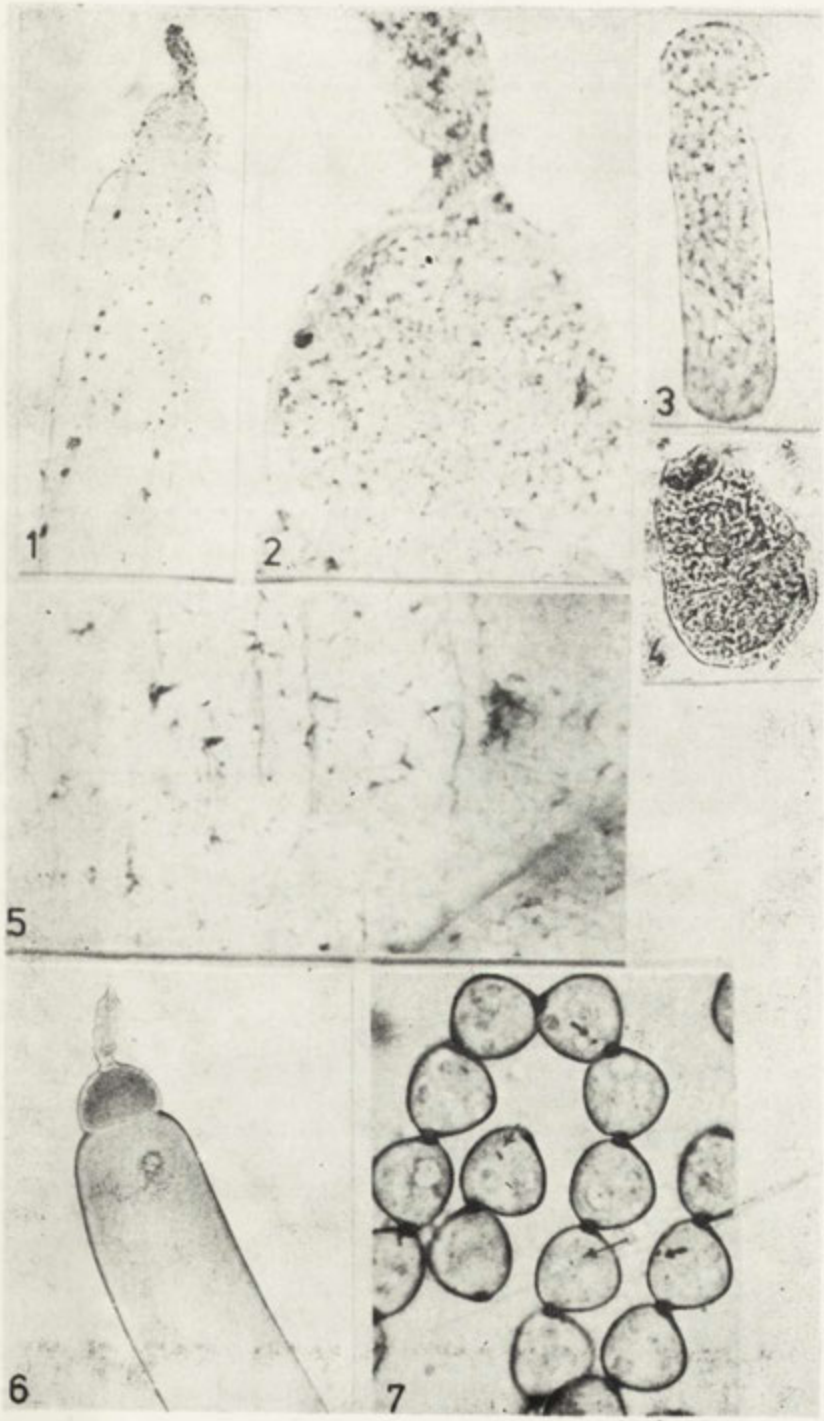
7: *S. conoides.* Spores, × 2000. Note traces of enzyme activity (arrows)

8: *S. conoides.* Anterior portion of a trophozoite incubated in the substrate medium at pH 2.9, × 200. Note the near absence of enzyme activity even in the epimerite

9: *S. conoides.* Anterior portion of a trophozoite from the host that starved for 72 h, × 200. The enzyme activity is generally weak

10: Anterior portion of a trophozoite, × 400

11: Magnified portion of the trophozoite's ectoplasm × 1000. Note distinct deep brown granules indicating the enzyme activity



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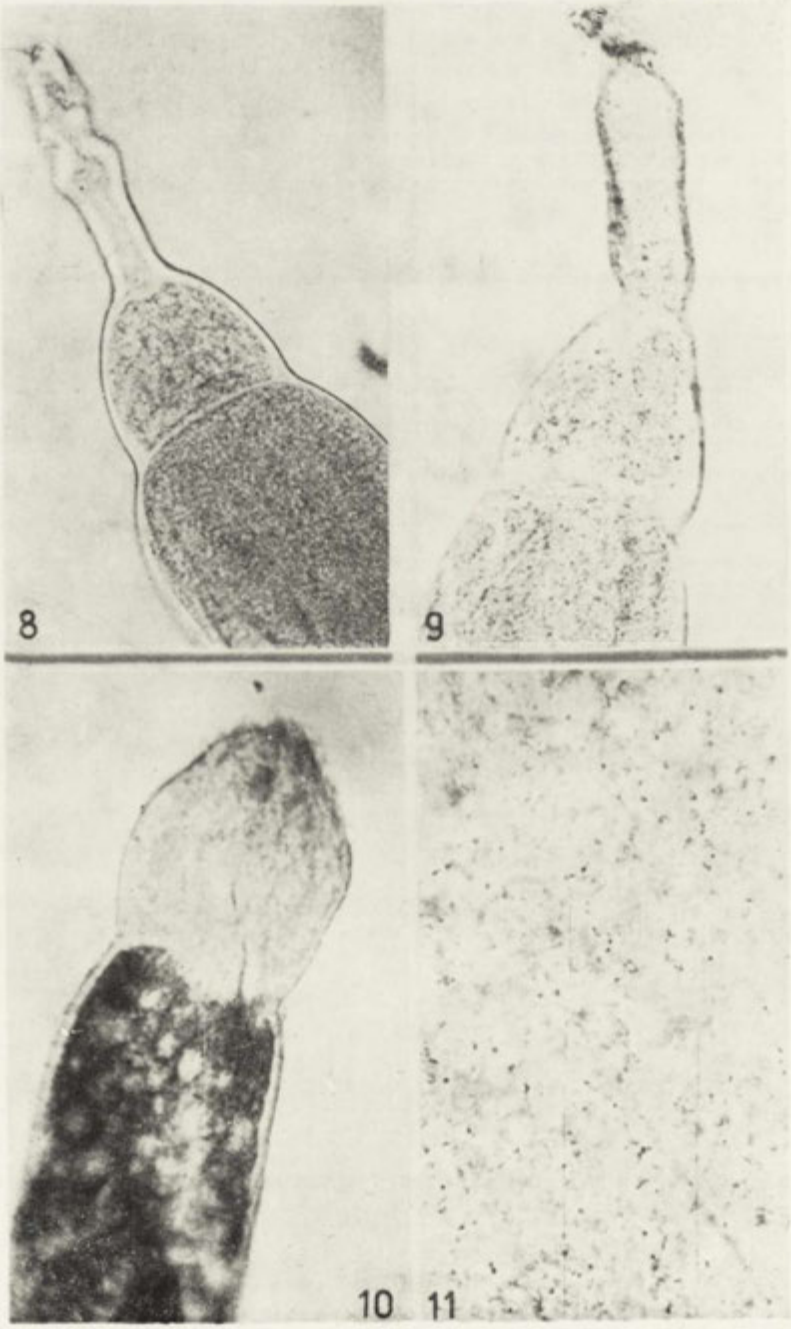

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Pleatospora termitis gen. nov., sp. nov. An Eugregarine Parasite of *Macrotermes estherae* (Desnaux)

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Synopsis. The morphology and life history of a new species of cephaline gregarine (*Apicomplexa: Sporozoea*) have been described. Since the observed features of this gregarine did not alley with any of the known genera and species, a new genus, *Pleatospora* is proposed under the family *Hirmocystidae Grasse*. The gregarine is named as *Pleatospora termitis* gen. nov., sp. nov.

Termites have been reported to be infested with both acephaline (Henry 1933, Desportes 1963 and Kalavati 1977, 1979) and cephaline (Leidy 1881, Ellis 1913, Kirby 1927, Ghidini and Moriggi 1941, Desai and Uttangi 1961, 1962 a, b, Uttangi and Desai 1961, 1962, Gisler 1967, Huger and Lenz 1976, Kalavati and Narasimhamurti 1978, 1980 and Theodorides et al. 1976) gregarines from various parts of the world. It appears from the literature that to date about 50 gregarine species including three acephaline ones have been described from termite hosts. These belong to genera *Gregarina* Dufour (*Gregarinidae* Labbe), *Hirmocystis* Labbe (*Hirmocystidae* Grasse), *Sphaerocystis* Leger (*Spherocystidae* Chakravarty), *Steinina* Leger and Duboscq (*Actinocephalidae* Leger emend Grasse), *Monocystis* Stein (*Monocystidae* Bütschli), *Anthorhynchus* Labbe (*Actinocephalidae* Leger emend Grasse), *Actinocephalus* Stein (*Actinocephalidae* Leger emend Grasse), *Kofoidina* Henry (*Lecudinidae* Kamm) and *Diplocystis* Schneider (*Diplocystidae* Schneider).

During the present study undertaken to evaluate the microbial flora and fauna of various termites prevailing in Gulbarga, authors came across a species of gregarine in the gut of *Macrotermes estherae* (Des-

naux), which by possessing unique oocysts could not be assigned to any of the known genera. As a result a new genus under the family *Hirmocystidae* Labbe is proposed to include it.

Material and Methods

Various castes of the termite were collected from mounds located at different places in and around Gulbarga University Campus, Gulbarga. These were brought to laboratory and dissected under dissecting microscope to take out the entire gut. For the examination of gregarines, the infected gut was teased in insect ringer solution on a glass slide. Smears were prepared using the contents of the gut from different regions. After air drying, the smears were fixed in Carnoy's fluid and stained with iron alum haematoxylin. For the study of intracellular stages, if any, the heavily infected gut was fixed in alcoholic Bouin's fluid, sectioned at 5-6 μm thickness and stained as above. The gametocysts collected from the gut were kept in moist chamber and examined at intervals for gametogenesis and sporogony.

The illustrations given in the paper are made with the help of camera lucida. Various morphometric measurements are made with the aid of calibrated eyepiece.

Observations

The percentage of infection among termite workers is 40 while in both minor and major soldiers it is about 30. Maximum infection prevails during months of October and November. Description of various stages in the life cycle of the gregarine is as follows:

Trophozoites. The gregarine appears to have no intracellular stage of development as the histological preparations of the gut did not reveal it. The early cephalont stage (Fig. 1 *A*) was observed in smears of the foregut. It is three-segmented one with ovoidal deutomerite, dome-shaped protomerite and a sessile simple hemispherical epimerite. The nucleus is large and ovoidal in shape. During the later stages of development, the early gamont's deutomerite enlarges enormously assuming cylindrical shape (Fig. 1 *B*, *C*). After the epimerite is shed off, the protomerite assumes hood-shaped structure. The nucleus becomes irregularly ovoidal.

Various dimensions of the early cephalonts are:

	Range	Mean
	in μm (of 25 specimens)	
Total Length	47-304	119.00
Epimerite Length	5-30	12.92
Epimerite Width	8-38	14.38
Protomerite Length	8-18	14.3

Protomerite Width	15-40	20.84
Deutomerite Length	26-256	186.76
Deutomerite Width	10-50	26.23
Nuclear Length	5-30	13.41
Nuclear Width	4-17	9.08
PW:DW ratio	1:1-1:1.75	1:1.19
PL:TL ratio	1:4.2-1:25	1:9.35

Gamonts. Two late cephalonts after shedding off the epimerite get attached caudofrontally and become biassociative forms (Fig. 1 *D*). Both the primite and satellite are elongated cylindrical in shape. Such forms measure 147-714 µm in length. The nucleus in them always occupies the middle of the deutomerite. The various measurements of both primite and satellite are:

	Primite		Satellite	
	Range	Mean	Range	Mean
	in µm (of 25 specimens)		in µm (of 25 specimens)	
Total Length	88-339	227.34	59-375	230.00
Protomerite Length	13-55	35.18	10-40	18.56
Protomerite Width	10-60	34.8	8-55	34.78
Deutomerite Length	75-284	198.78	47-335	211.5
Deutomerite Width	10-110	49.87	10-115	44.31
Duclear Length	5-45	21.94	7-30	19.36
Nuclear Width	5-25	14.68	4-22	12.88
NW:DW ratio	1:1-1:2.33	1:1.46	1:1-1:2.16	1:1.51
PL:TL ratio	1:2.8-1:18.14	1:7.58	1:5.6-1:17.6	1:10.49

Gametocysts. Mature biassociative gamonts roll up on each other and become gametocysts. These are broadly oval in shape measuring 300-370 µm in length and 270-290 µm in breadth. In freshly formed gametocyst (Fig. 1 *E*) two gamonts enclosed in a smooth and thin cyst wall could be seen. Isogametes are formed on third day which is evidenced by the disappearance of partition wall. These cysts mature (Fig. 1 *F*) within 6-10 days and release oocysts by simple rupture.

Oocysts. These are cylindrical in shape and are released singly. The oocysts wall folding on itself is modified into three rectangular plates or ridges running all along its length (Fig. 1 *G*). In frontal view these plates appear like triangular cones arranged around the central ring (Fig. 1 *H*). In lateral view plates appear like three wings of the oocysts.

Systematic Position

The diagnostic features of the gregarine such as the presence of protomerite and deutomerite, septum, biassociative sporadins, simple knob-like epimerite, gametocysts dehiscing by simple rupture justify

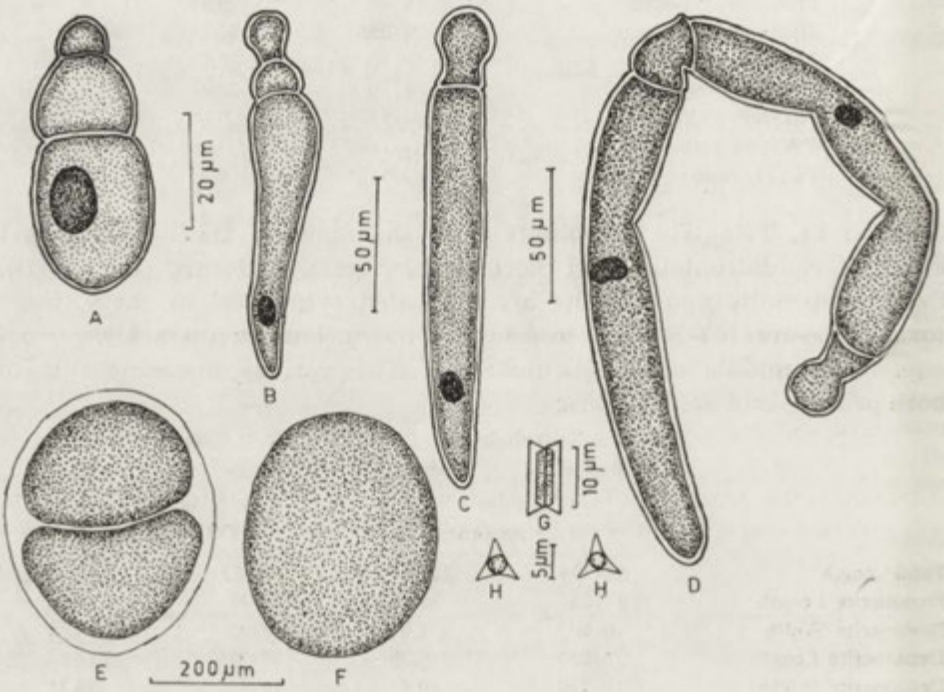

Fig. 1. A-H Camera lucida drawings of *Pleatospora termitis* gen. nov., sp. nov., *A* — Early cephalont (trophozoite), *B* — Late cephalont, *C* — Trophozoite without the epimerite, *D* — Biassociative gamont, *E* — Freshly formed gametocyst with partition wall, *F* — Mature gametocyst, *G* — Lateral view of cylindrical oocyst. Note the pleat-like rectangular plates, *H* — Frontal view of the oocyst. Note the triangular arrangement of plates

the inclusion of it in the family *Hirmocystidae* Grasse (*Apicomplexa* : *Sporozoea*).

The family *Hirmocystidae* includes nine genera which are characterized by possessing either ellipsoidal, prismatic, fusiform, ovoidal or evenly spherical oocysts (Chakravarty 1959). None of these genera has plate bearing oocysts. The only genus thus far known to possess winged oocysts is *Alaspora* Obata (1953) which belongs to entirely different family i.e., *Actinocephalidae* Leger emend Grasse. Since the gregarine under report possesses characters agreeing with the family *Hirmocystidae* but differ from the known genera by possessing winged oocysts, a new genus, *Pleatospora* is created to assign this gregarine, *P. termitis* gen. nov., sp. nov., a taxonomic status. The generic nane is proposed to denote pleat-like folds of the oocyst's envelope assuning three wing-like structures. While, the specific name signifies the termite host in which the gregarine has been found.

Diagnosis. The diagnostic features of the genus *Pleatospora* gen. nov. are as under:

(1) Sporadins biassociative.
(2) Epimerite simple globular knob.
(3) Gametocysts without sporeducts, dehisce by simple rupture.
(4) Oocysts cylindrical with rectangular wings or plates running all along the longitudinal axis.
(5) Gut parasites of termites.

Type species: *Pleatospora termitis* gen. nov., sp. nov.
Host: *Macrotermes estherae*, (Desnaux).
Locality: Gulbarga University Campus, Gulbarga, Karnataka, India
Site of Infection: Fore and midgut.
Repository: Department of Post Graduate Studies and Research in Zoology, Gulbarga University, Gulbarga 585 106.

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Myxosoma indirae sp. n. (*Myxozoa : Myxosomatidae*) from the Head Cartilage, Scale and Tail Fin of *Cirrhina mrigala* (Hamilton)

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Synopsis. A myxosporidian parasite, *Myxosoma indirae* sp. n. (*Myxozoa : Myxosomatidae*) has been described from the head cartilage, scale and tail fin of a fresh water teleost, *Cirrhina mrigala* (Hamilton). The dimensions of its spores and affinities with the related species have been discussed. A comparison of other *Myxosoma* spp. reported from the head cartilage of other fishes have also been incorporated.

The genus *Myxosoma* Thélohan, 1892 (*Lentospora* Plehn, 1905) is characterized as having circular, oval or ellipsoidal spores, two pyriform polar capsules and without an iodinophilous vacuole. Several species have been described under this genus in recent years by several workers from the different parts of the world. During my survey work on the myxosporidian parasites from the edible fishes of West Bengal, I have come across a parasite of the genus *Myxosoma* Thélohan from the head cartilage, scale and tail fin of *Cirrhina mrigala* (Hamilton).

Material and Methods

The fishes, *Cirrhina mrigala*, were collected from the local ponds at Ranaghat, about 70 km north of Calcutta and were brought to the laboratory. These were immediately examined for presence of myxosporidian parasites under the binocular (dissecting and compound) microscopes. Cysts of the myxosporidan were found within the scale and tail fin of the infected fishes. Examination of fresh spores obtained from the cyst smears and as well as from the smears of brain meninges and cartilaginous parts of cephalic regions were made with a drop of

normal saline. Some of the smears were treated with Lugol's iodine for the detection of iodinophilous vacuole, a few others were treated with 10% KOH solution for filament extrusion. The simple India-ink technique (Lom and Vávra 1963) was employed to reveal the presence of mucous envelopes. Air-dried smears were stained with Giemsa after fixation in absolute methanol. Illustrations were made with the aid of a camera lucida.

Observations

Myxosoma indirae sp. nov.

Type host: *Cirrhina mrigala* (Hamilton).
Locality: Ranaghat, West Bengal, India.
Infection locus: Cartilage of the head skeleton, scale and tail fin.
Prevalence: Eight infected out of 45 fish examined, in June 1983.

Cysts: Small, egg-shaped to spherical white bodies measuring 0.5 to 1.0 mm.

Developmental stages: Not found.

Spore: Broadly oval to spherical in front view (Fig. 1 *1-2*), lenticular in side and posterior end view (Fig. 1 *3-4*), suture thick, ridged and slightly oblique (Fig. 1 *3*), shell valves thick, smooth and without any striations, two equal polar capsules pyriform and convergent, 8 to

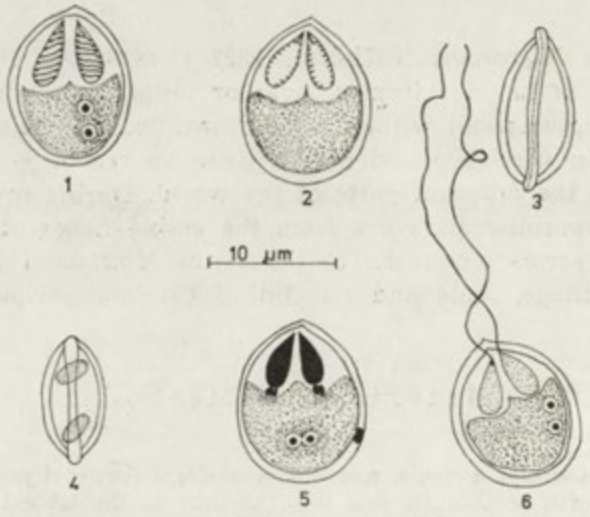

Fig. 1. *1-6*. Camera lucida drawing of *Myxosoma indirae* sp. n. *1* — A fresh spore in front view, *2* — A spore in front view treated with Lugol's iodine, *3* — A fresh spore in side view showing thick, oblique and ridged suture, *4* — A fresh spore in end view, *5* — An immature spore showing six nuclei — stained with Giemsa, *6* — A spore showing the polar filaments (after extrusion) — stained with Giemsa

10 coils of polar filament in each capsule, sporoplasm crescentic, finely granulated and binucleate filling the whole of the extra-capsular region, iodinophilous vacuole absent, in developing spores (Fig. 1 5) in addition to 2 sporoplasmic nuclei, 2 capsulogenous and 2 valvular nuclei present, polar filaments large, equal and of uniform thickness (Fig. 1 6), mucous envelope absent.

Pathogenicity: Apparently none.

Measurements (in microns) (based on 45 fresh spores):

Spore
Length — 12.6 (11.0-14.0), Breadth — 9.6 (9.0-11.0),
Thickness — 2.4 (2.2-3.0),
Polar capsule
Length — 4.7 (4.0-6.0), Breadth — 2.2 (2.0-2.5),
Polar filament
Length — 31.6 (28.0-37.0),
Nucleus — 1.7 (1.5-2.0).

Material: Syntype specimens on slide No. TC/M-1, kept in the Department of Zoology, Ranaghat College, will shortly be submitted to the National Collection of the Zoological Survey of India, Calcutta.

Discussion

A survey of literature reveals that myxosporidian parasites have been reported from various organs of a number of fishes in India (Tripathy 1952, Lalitha Kumari 1969). Of these, only twelve belonging to the genus *Myxosoma* (Hofer) have been described from India. Six *Myxosoma* spp. have so far been reported from the cartilage of various fishes (Table 1). Amongst them, *M. cerebralis* (Hofer) (cited from Lom and Hoffman 1971), *M. cartilaginis* Hoffman et al. 1965, *M. filamentosa* Haldar et al. 1981 and *M. gangulii* Sarkar et al. 1982 have been described from the cartilage of head skeleton whereas *M. scleroperca* Guilford, 1963 (cited from Hoffman et al. 1965) and *M. hoffmani* Meglitsch, 1963 have been reported from the cartilage of the sclera of the eye. A comparison of the present species with those mentioned above shows its close resemblance with *M. cerebralis* in the general shape of the spore. However, the spore of *M. cerebralis* is much smaller in dimension from the present species. It also resembles *M. hoffmani* Meglitsch, 1963 in the number of polar filament coils within the polar capsule and in the dimension of the polar capsule but differs distinctly from the latter in location (the latter is found only in the sclera of the eye) and also in the spore dimension. The species under

Table 1

The comparative spore dimensions (microns) of *Myxosoma* spp. reported from the cartilage of fishes

Specification	*M. cerebralis*	*M. cartilaginis*	*M. filamentosa*	*M. hoffmani*	*M. scleroperca*	*M. gangulii*	*M. indirae* sp. n
Spore							
Length	8.7 (7.4-9.7)	10.8 (10.0-12.0)	13.7 (11.2-17.3)	9.3 (8.6-10.8)	10.0-19.2	8.73 (8.0-10.0)	12.6 (11.0-14.0)
Width	8.2 (7.0-10.0)	9.5 (9.0-11.0)	9.5 (8.1-12.2)	8.4 (7.8-8.9)	7.2-9.6	5.03 (4.8-6.5)	9.6 (9.0-11.0)
Thickness	6.3 (6.2-7.4)	6.1 (6.0-7.0)	–	6.2 (5.9-6.5)	6.0-9.6	4.6 (3.5-5.5)	2.4 (2.2-3.0)
Polar capsule	Equal	Equal	Equal	Equal	Unequal	Unequal	Equal
Length	5.1 (5.0-6.0)	5.3 (5.0-6.0)	5.8 (4.0-7.1)	5.0 (4.6-5.7)	10.8 or 9.5	3.6 or 2.4	4.7 (4.0-6.0)
Width	3.2 (3.0-3.5)	3.1 (3.0-4.0)	3.1 (2.0-4.0)	2.4 (2.2-2.7)	2.4 or 3.6	1.9 or 1.5	2.2 (2.0-2.5)
Polar filament	5 to 6 coils	5 to 7 coils	5 to 6 coils	10 coils	–	5 to 6 coils	8 to 10 coils
Length	–	–	29.3 (23.4-34.6)	–	–	–	31.6 (28.0-37.0)
Infection locus	Cartilage of head skeleton	Cartilage of head skeleton and gill arch	Cartilage of head skeleton and meninges	Cartilage of sclera of eye	Cartilage of sclera of eye	Cartilage of head skeleton	Cartilage of head skeleton, scale and tail fin
Reference	Lom and Hoffman (1971)	Hoffman et al. (1965)	Haldar et al. (1981)	Meglitsch (1963)	Hoffman et al. (1965)	Sarkar et al. (1982)	Present study

consideration is unique in the sense that it has been observed from the head cartilage, scale and tail fin of the same fish without any marked morphological or morphometrical variations. The parasite is therefore, considered to be a new species and the name *Myxosoma indirae* sp. n. is proposed after the Late Prime Minister, Mrs. Indira Gandhi.

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Spontaneous Sarcocystosis in Indigenous Goats in Bihar, India

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Synopsis. Preliminary studies, based on the examination of 47 meat samples from goats slaughtered at local abattoirs of Patna (India), revealed moderate (++) to heavy (+++) infection in cardiac and oesophageal musculature. However, none of the samples of ocular muscles were found infected with sarcocysts. Organwise, microform (smaller species) of sarcocysts (*Sarcocystis capracanis*) were observed in 12 of 27 heart samples (44.4%) and five of 12 tissue samples (41.6%) of oesophageal muscles. Intensity of infection with sarcocysts was greater in the heart than the oesophagus. Of the infected animals, goats exhibited 41.1% each of light (+) and moderate (++) degree of infection while only 17.5% showed heavy (+++) infection of sarcocysts in their muscles. The sarcocysts were morphologically indistinguishable from those of *S. capracanis* (Fischer 1979). Morphological variations in the shape and size of the sarcocysts from samples of heart and oesophageal musculature were fairly distinct. Sarcocysts isolated from cardiac muscles were predominantly maggot-like to spindle-shaped and contained spherical to banana-shaped merozoites with rounded tips at both the ends in their cystic cavity. The freshly collected cysts measured 275 to 475 μm in length and 75 to 137.5 μm in width. In contrast, the cysts isolated from oesophageal muscles were comparatively thinner and elongated with one end pointed and the other rounded and measured 550 to 587.5 μm in length and 50 to 75 μm in width. The infected goats did not exhibit any detectable febrile clinical symptom of sarcocystosis during antimortem examination.

Parasites later described under the genus *Sarcocystis* Lankester, 1882 were first discovered from striated muscles of rodents by Miescher (1843). Subsequently, it was reported from a wide range of host species e.g., cattle, buffaloes, sheep, goats, deer and other closely related antelopes from different parts of the world (Jadros and Laar-

man 1982). This parasite causes a syndrome which is popularly described as sarcosporidiosis or sarcocystosis and is characterized by muscular atrophy, loss in productivity, anorexia, abortions in pregnant herbivorous hosts and diarrhoea, loss of appetite and digestive disturbances in the canine or feline definitive hosts. Predominatly, two forms of the disease were reported both from herbivorous animals and from human beings, i.e., the muscular or tissue form and the intestinal form, respectively. Unlike coccidian parasites, *Sarcocystis* requires two obligatory hosts for the completion of its life cycle. The sexual stages (gamonts and gametes) develop in the intestinal tract of carnivorous animals (dogs, cats, jackals, foxes etc.) and are excreted as sporocysts in their faeces. Asexual stages (masonts and sarcocysts) are formed in the endothelium of blood vessels and muscles of various internal organs of herbivorous animals (cattle, buffaloes, sheep, goats, deer, etc.). All the species of *Sarcocystis*, known so far, exhibit two generations of zoites within the cysts, i.e., metrocytes and merozoites. However, the presence of three cyst forms e.g., metrocytes, merozoites and intermediate stages have also been reported recently (Beyer et al. 1981).

Caprine sarcocystosis generated a great interest among the parasitologists recently after the classical work of Fischer (1979) on *S. capracanis* and a spate of recent publications from all over the world (Collins and Crawford 1978, Aryeetey et al. 1980, Dubey 1983 a, b, Rao and Rao 1983). The present study was thought to be of interest because of the recent reports of the association of some *Sarcocystis* sp. in causing muscular form of human sarcocystosis (Rommel and Heydorn 1972, Pathmanathan and Kan 1981) and also because goats are predominantly undisputed meat animals for a majority of the Indian population.

Materials and Methods

The Materials

Tissues from different organs like the heart, oesophagus and eye were collected from goats slaughtered at the local abattoirs. In addition, materials were also collected during postmortem examination of the animals brought to the postmortem hall of Bihar Veterinary College, Patna, India. Muscle tissues of suitable sizes were cut with sharp scissors from organs like oesophagus, heart and eye etc. The cysts were collected in buffered physiological saline in specimen jars and isolated from the adjoining fascia and other muscular structures with the help of fine pointed forceps. Approximately half of the tissue samples of each organ was removed out in physiological saline solution and examined under a dissecting microscope for detecting intramuscular sarcocysts using the methods described by Sahai et al. (1983).

The Parasite

Intramuscular cysts obtained from the cardiac and oesophageal muscles were washed thrice with physiological saline solution and examined unstained in watch glasses for the study of their shapes and sizes. Morphological variations of the sarcocysts isolated from different organs were recorded and measurements of freshly isolated unstained cysts were taken with the ocular micrometer.

Evaluation of Results

The intensity of infection with intramuscular sarcocysts was evaluated by conting the total number of cysts removed from one gram of the infected muscle sample. The presence of less than 15, between 16-50 and over 50 microcysts was classified as light (+), moderate (++) and heavy (+++) intensities of infection, respectively.

Clinical observations were also made during antimortem examination of goats prior to their slaughtering.

Results

Data on the occurrence of *Sarcocystis* infection in goats have been summarized in Table 1. It is evident that of the total 47 samples (27 cardiac, 12 oesophageal and 8 ocular muscles) examined, 17 samples (12 cardiac, 5 oesophageal) were positive for sarcocysts of *S. capracanis* (smaller cyst forms having dogs as definitive host). However, in none

Table 1

Organwise prevalence of *Sarcocystis capracanis* infection in goats

Sl. No.	Organs/Tissues	Samples examined	Samples positive	Percent of infection
(1)	Cardiac musculature	27	12	44.4
(2)	Oesophageal musculature	12	5	41.6
(3)	Ocular musculature	8	—	—
	Total	47	17	36.6

of the meat samples, sarcocysts of *S. ovifelis* (the larger species) were detected. The incidence rate was marginally higher in cardiac muscles (44.4%) than in oesophageal musculature (41.6%), with an overall incidence rate of 36.6%. However, none of the samples of ocular muscles of goats examined, revealed the presence of sarcocysts. Statistical analysis of data recorded did not reveal any significant variation in the infection rate of cardiac and oesophageal musculature when tested with chi-square test ($\chi^2 1$ df = 0.033 NS).

Data on the intensity of *Sarcocystis* infection in the cardiac and oesophageal muscles are summarized in Table 2. It is evident that of the total 17 positive muscle samples (12 cardiac and five oesophageal), the intensity of infection of sarcocysts was light (+) in four (33.3%) heart and three (60.0%) oesophagus samples with the overall light (+)

Table 2

Average intensity of *Sarcocystis capracanis* infection in the musculature of the heart and oesophagus of goats

Sl. No.	Infected tissues	No. of positive organs	Intensity of infection		
			Light (+)	Moderate (++)	Heavy (+++)
(1)	Cardiac musculature	12	4 (33.3)	6 (50.00)	2 (16.6)
(2)	Oesophageal musculature	5	3 (60.0)	1 (20.00)	1 (20.00)
	Total	17	7 (41.1)	7 (41.1)	3 (17.6)

N.B. Figures in parentheses denote percentage of infection
Intensity of microcysts per cubic centimeter of the infected tissues
Light (+) — 1-14 microcysts
Moderate (++) — 15-49 microcysts
Heavy (+++) — 50 and above microcysts

infection rate of 41.1%. On the other hand, six (50.0%) heart and one (20.0%) oesophagus samples were found moderately (++) infected with the overall moderate (++) infection rate of 41.1%. The intensity of heavy (+++) infection was observed in only two (16.6%) heart and one (20.0%) oesophageal samples giving the overall heavy (+++) infection rate of 17.5%.

Detailed morphological studies of sarcocysts isolated from samples of both cardiac and oesophageal musculature revealed distinct variation in their shapes and sizes. While majority of the sarcocysts obtained from the heart samples were somewhat spherical and maggot-like in appearance, some of these were spindle shaped with rounded tips at one or both the ends. These cysts were filled up with spherical to banana-shaped merozoites in it. The cysts were white opaque in colour when examined fresh and were very difficult to detect in tissues with naked eye. However, such cysts were easily detectable under the dissecting microscope as lying on the long axis of the muscle fibres. The freshly collected unstained cysts measured 275 to 475 μm in length and 75 to 137.5 μm in width. The cyst wall was thick, striated, septate and divided the sarcocystic cavity into many compartments. Each cystic compartment was seen completely filled up with metrocytes and merozoites (Pl. I 1-3).

In contrast to the morphological features of sarcocysts from cardiac musculature, the cysts isolated from oesophageal tissues were thinner and elongated. These were opaque, white in colour with one end pointed and the other rounded. Freshly removed specimens of sarcocysts measured 550 to 587.5 μm in length and 50 to 75 μm in width. The cyst walls were thin, striated and septate but the septation was comparatively less distinct. However, in the samples of oesophageal musculature too, the sarcocystic cavity, was found completely filled up with metrocytes and mature merozoites (Pl. II 4-6).

Clinical observations recorded during the antimortem examination of goats prior to slaughtering did not reveal any detectable febrile symptom of high rise in body temperature, anaemia or emaciation.

Discussion

The data presented in Table 1 and 2 provided convincing evidence that caprine sarcocystosis is not uncommon in this part of the Bihar state (India) although the infection rate was not as heavy as recorded from cattle and buffaloes in the same area (Sahai et al. 1983). In the present investigation, the infection rate was found to be only 44.4 and 41.6% in locally slaughtered goats in cardiac and oesophageal muscles, respectively. This is in contrast to the reports of only 28.3% by Collins and Crawford (1978) from New Zealand and a very heavy infection rate of 97.22 and 55.26% by Shah (1981) and Gupta (1983) from central and northern India, respectively.

The data presented in Table 1 also reveals that asexual stages of *S. capracanis* have special preference for the tender musculature of vital organs, e.g., heart and oesophagus than the other organs having coarse and fibrous tissues in predominance. Rommel et al. (1972) also found heavy infection in the oesophageal muscles in the case of *S. tenella* infection in sheep. In the present study, cardiac musculature exhibited the highest infection rate followed by oesophagus. However, unlike bovine sarcocystosis (Juyal et al. 1982) ocular musculature did not reveal the presence of *Sarcocystis* infection in goats examined during this study.

Morphological and biological characteristics of *Sarcocystis* sp. reported in the present study were broadly similar to the original descriptions of *S. capracanis* as described by Fischer (1979) and subsequent reports of Collins and Crawford (1978), Aryeetey et al. (1980) and Heydorn and Haralambidis (1982). However, the characteristic clinical symptoms of high rise in body temperature, di-

stinct anaemia and emaciation and the presence of two successive generations of schizonts observed by Fischer (1979) in experimental caprine sarcocystosis were not observed in any of the infected indigenous goat examined during the course of the present study. The absence of detectable febrile symptoms in the spontaneous sarcocytosis in indigenous goats observed in the present study are contrary to the previously reported high virulence of this species in experimental sarcocystosis (Leek and Fayer 1978, Fischer 1979, Heydorn et al. 1981, Dubey 1983 a, b). This sub-clinical to non-clinical nature of sarcocystosis in indigenous goats led us to believe that under natural conditions indigenous goats are comparatively more resistant to the pathogenic effects of *S. capracanis* than the exotic pure-bred goats and may harbour heavy load of *Sarcocystis* infection without exhibiting detectable syndromes of illness.

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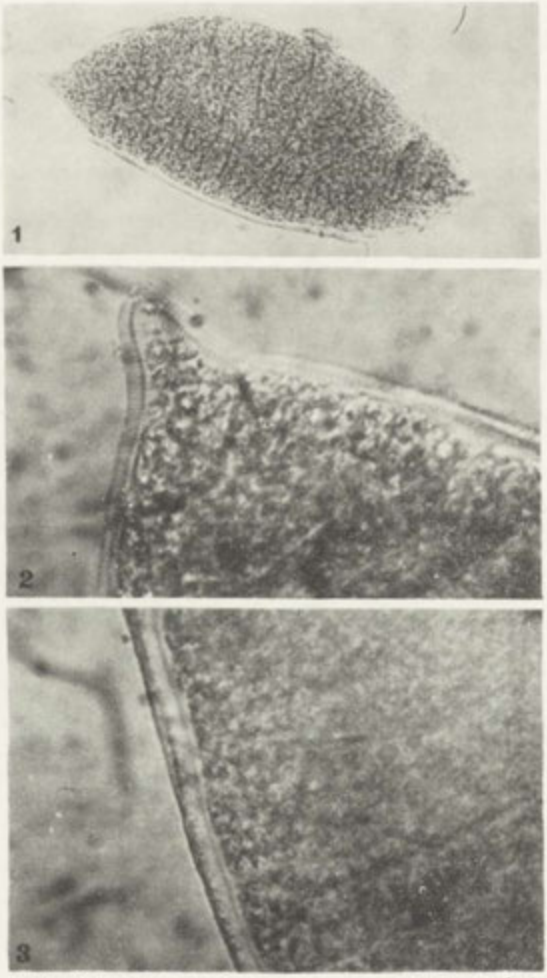
EXPLANATION OF PLATES I-II

1: Photomicrograph of a sarcocyst of *S. capracanis* from cardiac muscles of goats having spindle-shaped appearance, distinct septation with one end showing pointed projection and the other rounded × 63
2: Photomicrograph of the pointed end of a sarcocyst shown in phot. 1 showing distinct striations and thickness of the cyst wall × 400
3: Photomicrograph of a sarcocyst of *S. capracanis* showing thick cystic wall and spherical to banana-shaped metrocytes and merozoites in the cystic cavity × 400
4: Photomicrograph of a sarcocyst of *S. capracanis* from oesophageal muscles of goats having slender, elongated, work-like appearance with pointed ends × 19.16
5: Photomicrograph of a portion of a sarcocyst of *S. capracanis* from oesophageal muscles of goats showing thin cyst wall and less distinct septation × 63
6: Photomicrograph of a portion of sarcocyst of *S. capracanis* from oesophageal muscles of goats showing metrocytes and merozoites and thinner cystic wall × 400



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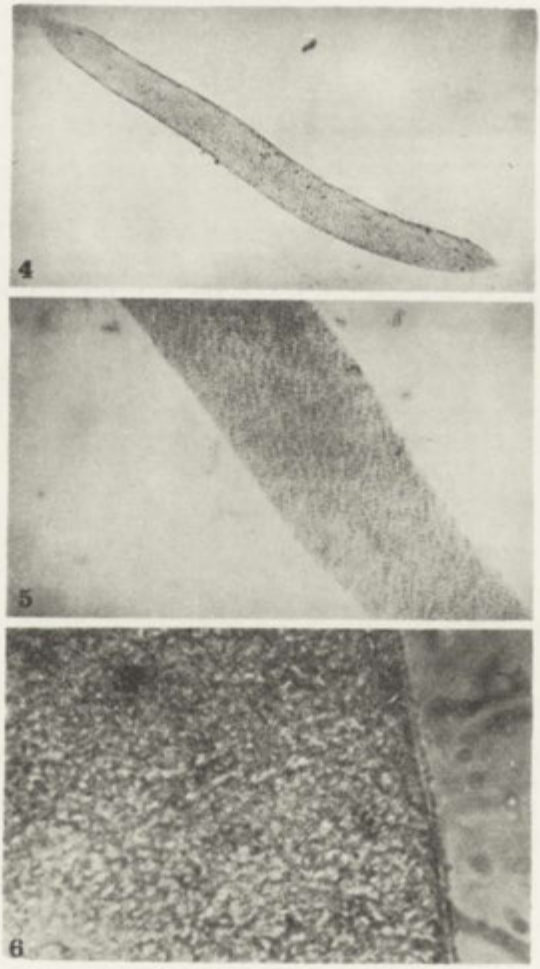

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