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The Ethogram of *Blepharisma japonicum* (*Protozoa, Ciliata*)

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Synopsis. The ethograms of vegetative organisms, heterotypic pairs, homotypic pairs and gigantic forms of *Blepharisma japonicum* show that this species creeps along piecewise linears, whose geometric elements (arcs and segments) do not follow each other completely at random and are joined to each other by three different types of motor reactions: (I) the Continuous Trajectory Change (CTC); (II) the Rough Trajectory Change (RTC) and (III) the Side-Stepping Reaction (SSR). The population is formed by two different types of organisms: (A) those creeping along segments and rightward arcs and (B) those creeping along segments and leftward arcs. The swimming pattern of this species follows a typical helicoid, which can be easily described in terms of pitch and radius.

Jennings (1906) studied the behaviour of the *Protozoa* from a naturalistic attitude, lost in the following decades: protozoologists examined the locomotion of *Protozoa* only in fact as a means of studying the fine mechanisms responsible for their motor activity, rather than as a natural phenomenon deserving attention per se. Thus, our information about the molecular bases of motility (cf. Machemer and Deitmer 1987) is far more abundant and exhaustive than that about the motility itself of *Protozoa* (Kung 1976, Van Houten et al. 1981).

Ethograms (sensu Eibl-Eibesfeldt 1967; Ricci 1986) so far established for Ciliates describe the motility of *Oxytricha bifaria* (*Ciliata, Hypotrichida*, Ricci 1981, 1982), *Euplotes crassus* (*Ciliata, Hypotrichida*, Ricci et al. 1987), *Litonotus lamella* (*Ciliata, Kynetophragminophora*, Ricci and Verni 1988), *Aspidisca* sp. (*Ciliata, Hypotrichida,*

This work was supported by grants from CNR and MPI.

Ricci et al. in prep.). In addition to answering the questions posed by Kung (1976) and Van Houten et al. (1981), the ethograms lend themselves to comparative studies (Ricci and Miceli 1983) and to experimental handling of populations of ciliates.

Blepharisma japonicum has been studied in order to produce another ethogram because: (1) the species belongs to a systematic group (Polyhymenophora) (Corliss 1979) not yet investigated from this point of view; (2) it is possible to induce different states of the same species (heterotypic pairs, homotypic pairs, giants) and to study their motility in comparative terms; (3) its biology is quite well known from greatly different points of view (cf. the book edited by Giese 1973, and the review of Miyake 1978), hence integration between this general knowledge and the ethological data specifically obtained for *B. japonicum* could provide a valuable instrument for new experiments and investigations, also from the point of view of its photobiology (Raab 1900, Giese 1946).

Methods

Two strains of *B. japonicum* (*Ciliophora, Heterotrichida*, Corliss 1979), T21 and R175, mating types I and II, respectively, were kindly supplied by Dr. A. Miyake of the University of Camerino. The cultures were kept in a dark room, in 1000 ml Erlenmeyer flasks, at 25-27°C and fed once a week. The lettuce medium, prepared according to Miyake's (1968) protocol, was inoculated with *Enterobacter aerogenes* 24 h earlier, under sterile conditions. Under proper conditions, cultures belonging to the different mating types were mixed and produced true heterotypic pairs (I-II) and homotypic pairs as well (I-I, II-II). The cell size and colour (larger and darker for T21 cells than for R175 cells) enabled us to recognize, isolate and study the 3 different types of pairs. To obtain the giants the cultures underwent 7-10 days starvation (Giese 1973). The motility of these different states of *B. japonicum* was studied by means of videorecording, obtained by a Hitachi TV camera and a Betamax C7 TV recorder, coupled to a Wild M420 stereomicroscope. The cells to be recorded (at a cell density of 40-50 cells/cm^2) were kept between a slide and a coverslip, the distance separating their surfaces being about 3 mm, observed with a light of 1350 ± 25 lux, at 25°C.

Four tapes (~8 h of recordings) were used to record the motility of this species: each recording session was individuated by the strain of cells used, temperature, magnification (6.3 ×) and date. Magnification of 32 × was used only to resolve the most complex reactions. Analysis of the geometrical elements describing a track was conducted on the photocopies of the transparancies, on which the tracks had been recorded by hand. Three geometrical elements can be distinguished along the tracks and described in terms of lengths, angles, spaces, amplitudes, etc.: rightward arcs (A+), straight segments (S), leftward arcs (A−). Three motor reactions join a geometrical element to the next: CTC (Continuous Trajectory Change), RTC (Rough Trajectory Change), SSR (Side-Stepping Reaction). This terminology corresponds to that already published by Ricci (1981, 1982), Ricci et al. (1987), Ricci and Verni (1988). The velocity of a certain cell was calcula-

ted from the length of a track, measured in μm, and the time taken by that *Blepharisma* to move along the same track: such time was determined by calculating the mean of three successive values of the time needed to cover a given distance.

Results

I — Behaviour of single cells

Creeping

B. japonicum creeps forwards along irregular piecewise linears, formed by segments and arcs, joined to each other by means of 3 different kinds of trajectory changes (Fig. 1). The elements of a track can be

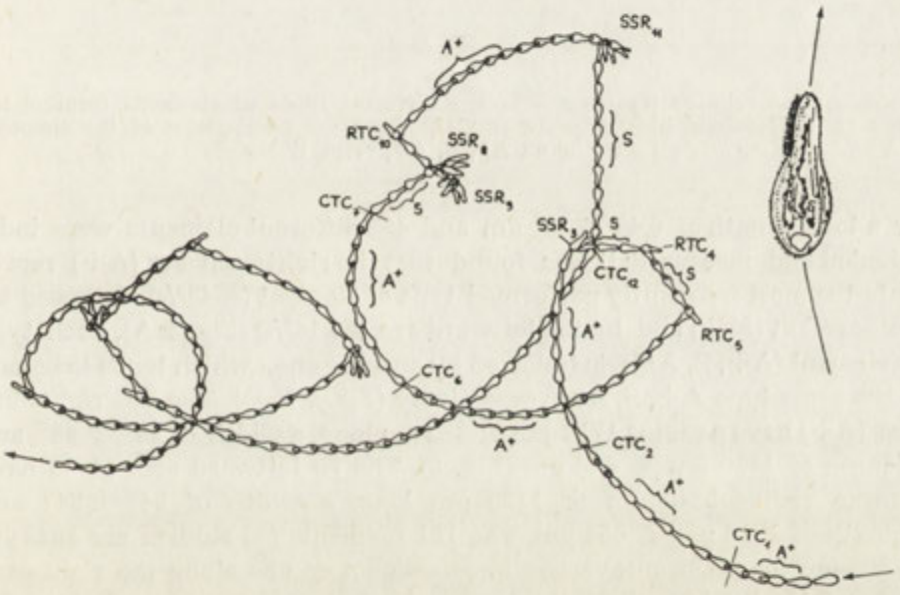

Fig. 1. The creeping track of *Blepharisma japonicum*. 12 motor reactions have been indicated, together with the geometric elements forming the track. CTC: Continuous Trajectory Change; RTC: Rough Trajectory Change; SSR: Side-Stepping Reaction; A+ : rightward arc; S: segment. The figure on the right shows that a creeping blepharisma has perioral apparatus oriented toward the left

described as geometrically perfect ($p < 0.001$) segments (S, straight tracks), rightward arcs (A+, with centers to the right of the organisms) and leftward arcs (A−, with centers to the left of the cell). *B. japonicum* includes two different types of organisms which keep the cytostome to the left (Fig. 1) and move forwards either along segments and rightward arcs (~65% of the population) or along segments and leftward arcs (~35% of the population). Tracks of 60 different cells were analyzed

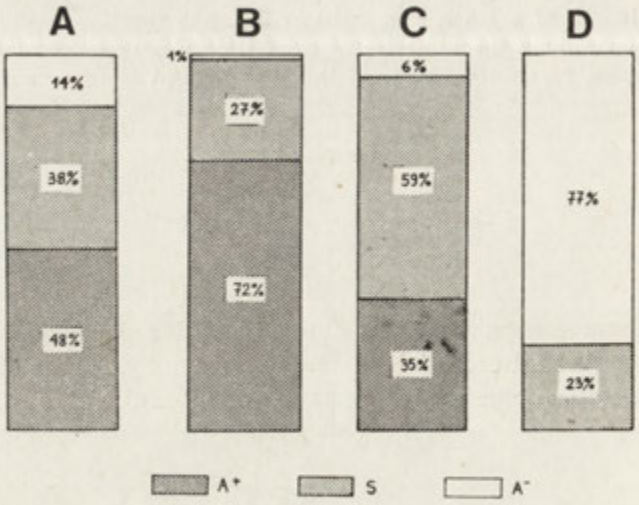

Fig. 2. A: the relative frequencies of the different types of elements forming the track of the creeping blepharismas. B, C, D show the frequencies of the elements following an A+, S, A−, respectively

for a total length of 6.43×10^5 μm and 423 different elements were individuated and measured: it was found that the rightward arc (A+) represents the most frequently performed type of element (~48%), followed by the segment (38%) and by the leftward arc (~ 14%) (Fig. 2 A). Each type of element (A+, S, A−) is followed by another one, which tends to belong to the same type A+, S, A− respectively (Fig. 2 B-D). The 202 rightward arcs (A+) have a radius 1718 μm ± 882 μm long, a width of 69° ± 48° and a length of 1750 μm ± 846 μm (Fig. 3). The 59 leftward arcs (A−) have a mean radius 2240 μm ± 1123 μm long, a width of 54° ± 34° and a length of 1996 μm ± 851 μm. The 161 segments (S) studied are 1033 μm ± 558 μm long. On an average *B. japonicum* creeps along the rightward arcs in 6.1″ ± 2.96″, along the leftward arcs in 7″ ± 4.1″ and along the segments in 3.8″ ± 2″. Once the proper ratios are calculated, the velocities of *B. japonicum* along rightward arcs, leftward arcs and segments are 287 μm/sec, 285 μm/sec and 272 μm/sec respectively, while the velocity in toto is 279 μm/sec ± 72 μm/sec (n = 60): it can also be expressed as 0.8 RU/sec, a Relative Unit being obtained by dividing the velocity by the average length of the species (350 μm). Each *B. japonicum* behaves characteristically, namely moving with fairly constant velocity along traits which tend to have approximately constant radii, lengths, widths etc.: the case of the radius has been chosen as an example and shown in Fig. 3, second graph. The arcs have radii and widths linearly correlated: (A+ : n = 202; y = 107 — 0.02 x; r = −0.50; $p < 0.001$; A− : n =

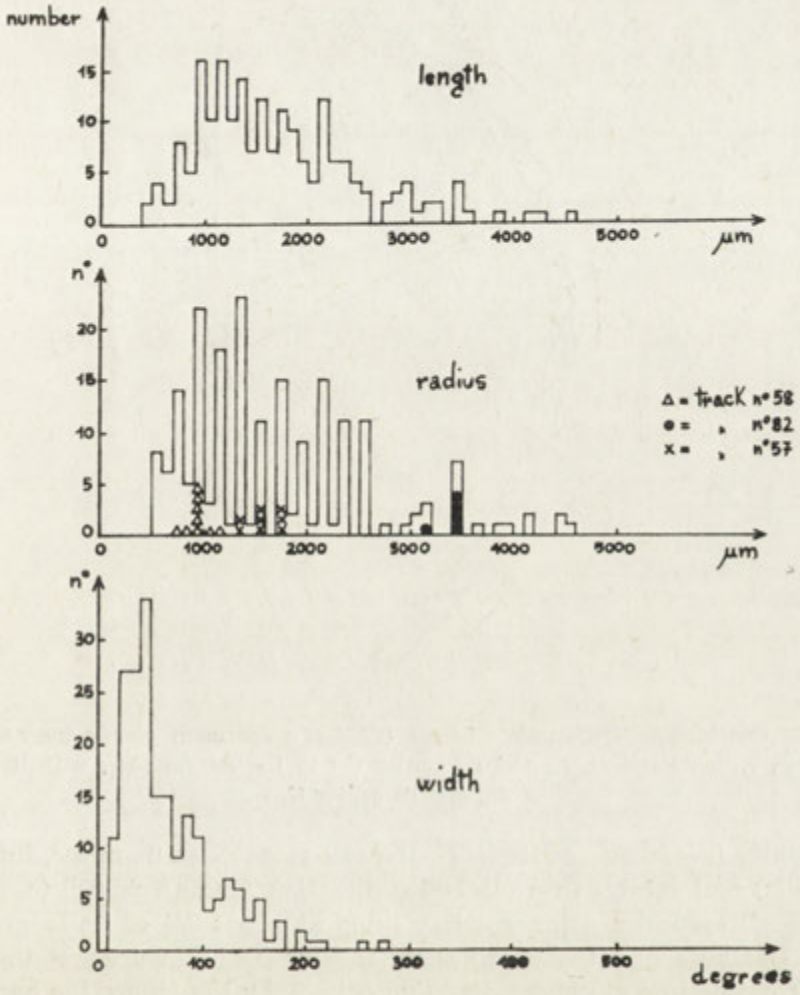

Fig. 3. A synopsis of the parameters characterizing the rightward arcs (A+). The mean length is 1750 μm ± 846 μm (n = 202); the mean radius is 1718 μm ± 882 μm (n = 202); the mean width is 69° ± 48° (n = 202). In the second histogram the values of the radii of three different organisms (n = 57, 58 and 81) are given, to show how constant they are for the same cell

59; y = 92 — 0.01 x; r = −0.42; $p < 0.001$) in such a way that as the radius enlarges, the width of the arc decreases.

Among the 437 reactions which affect the changes of trajectory for *B. japonicum*, three major types have been recorded: the CTC (n = 158: 36% of the cases), the RTC (n = 141: 32% of the cases), and the SSR (n = 138: 32% of the cases). By a CTC a *Blepharisma*, without slowing down, changes the direction of its creeping only slightly ($\alpha° = 4° \pm 13°$; n = 158) (Fig. 4). The RTC connects two succesive arcs with a sharp dis-

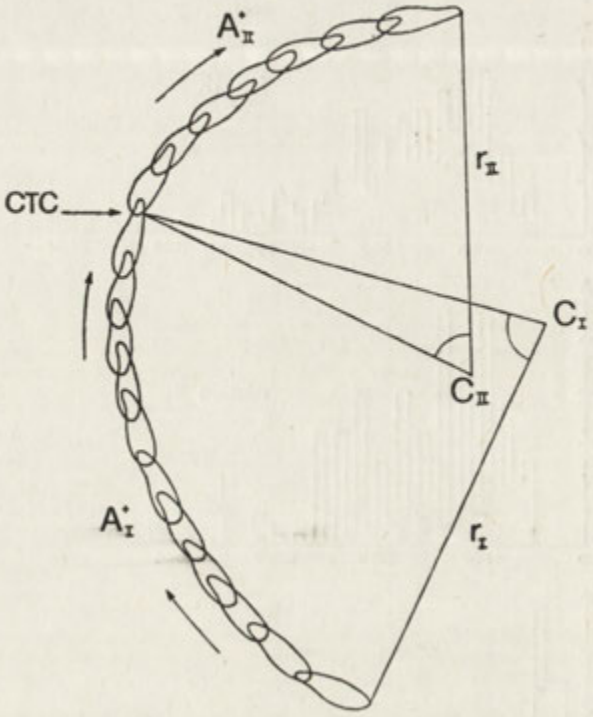

Fig. 4. Continuous Trajectory Change (CTC): a specimen passes from the first arc (A_I^+, with its radius, r_I, and its center C_I) to the second (A_{II}^+, with its radius, r_{II}, and its center C_{II})

continuity (Fig. 5 A): at its level, the cell stops ($\vec{v} = 0$ μm/sec, for about 1/10 sec) and rotates in a clockwise direction for 54° ± 30° (n = 140) (Fig. 5 C) around a point, roughly lying at about 3/4 of its length (Fig. 5 B). During an SSR, the cell stops and moves backwards, at the same time rotating in a clockwise direction (Fig. 6 A): the longer the backward motion, the wider the angle (Fig. 6 B). The length of the backward motion and the width of rotation are linearly correlated (n = 42; y = 50 + 1.08 x; r = 0.72; $p < 0.001$). While the general pattern of such rotation is quite unmistakable (Fig. 6 A), its quantitative aspects show somewhat wide distribution: the backward motion is 155 μm ± 62 μm (n = 43) and the clockwise angle is 101° ± 37° (n = 138) (Fig. 6 C). Fig. 7 shows the quantitative data for CTC, RTC and SSR.

Swimming

B. japonicum swims forward clockwise, along a geometrically perfect helicoid, keeping its cytostome toward the external surface of the cylinder containing the helicoid itself and the dorsal surface almost on the cen-

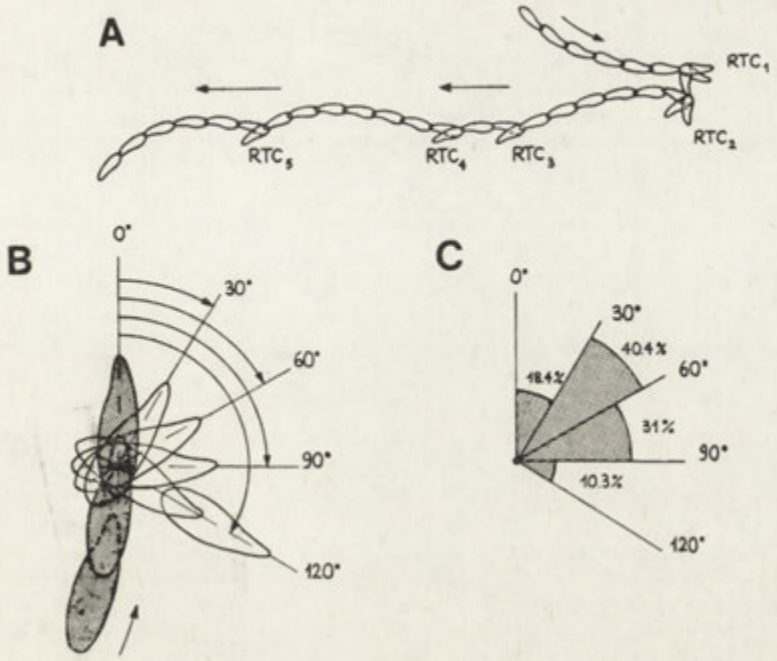

Fig. 5. (A) Five successive RTC of track n. 23; (B) each RTC consists of a clockwise rotation of the organism around a point, roughly lying at 3/4 of its length; (C) shows the distribution (%) of the different values of the angles

tral, straight axis of the same helicoid (Fig. 8 A): its pitch is 981 μm ± 289 μm long, and its radius is 74 μm ± 16 μm. Velocity is 388 μm sec ± 33 μm sec (1.1 RU/sec ± 0.09 RU/sec). The radius and the pitch of the helicoid are linearly correlated ($n = 28$; $y = 187 \pm 10.6\ x$; $r = 0.60$; $p < 0.001$). The reaction by which a *B. japonicum* changes its swimming direction consists of three successive steps (Fig. 8 B): (a) a sudden stop, (b) counterclockwise rotation around the posterior tip of the cell of about 180 degrees, (c) resumed swimming activity, in the new direction, lying roughly at about 90°-100° from the previous one.

II — The behaviour of heterotypic pairs

Creeping

The heterotypic pairs of *B. japonicum* are slightly asymmetric and the longer and darker partner, belonging to the type I cells, always lies on the right of the creeping pair: this stereodisposition must be exstremely convenient for the pair, because whenever it is lost (usually as a response to violent stimulation, such as bumping against other pairs, for

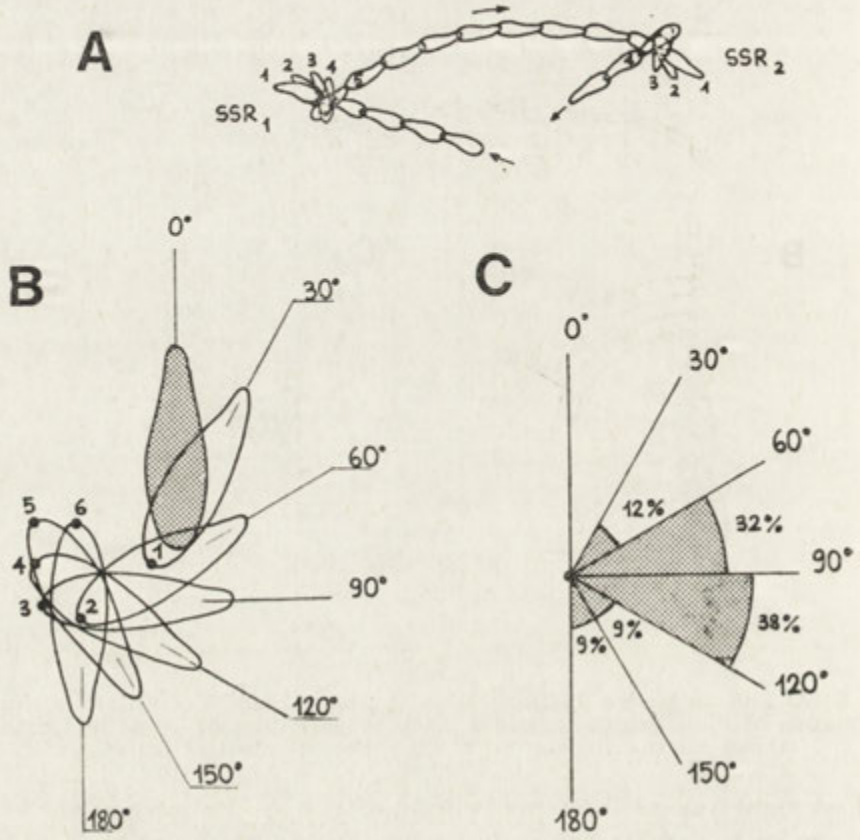

Fig. 6. Two SSR from the track n. 67 are shown in the scheme A, while B illustrates the backward motion and the simultaneous clockwise rotation; in C the percentage distribution of the different classes of angles is given

instance) it is reacquired rapidly, by means of an opportune countermaneuvre (Fig. 9). Each pair creeps along a piecewise linear formed by segments, leftward and rightward arcs; 29 tracks have been studied and 219 elementary traits recognized: 8 were A+ (= 3.6%), 64 were S (= 29.2%) and 147 were A− (67.2%). Thus, a heterotypic pair tends to move preferentially along leftward arcs, which have average lengths of 1514 μm ± 528 μm, radii of 1099 μm ± 481 μm and widths of 89° ± 45°, and are performed in about 9.7″ ± 4.8″. The rightward arcs have lengths of 1093 μm ± 180 μm, radii of 368 μm ± 268 μm and widths of 64° ± 17° and are performed in about 6.4″ ± 1.7″. The segments are, on an average, 877 μm ± 293 μm long and are covered by the pair in 7.5″ ± 4″. A highly significant negative linear correlation occurs between radius and width of the A−: the longer the radius the more narrow the angle ($n = 147$; $y = 113 - 0.02\,x$; $p < 0.01$).

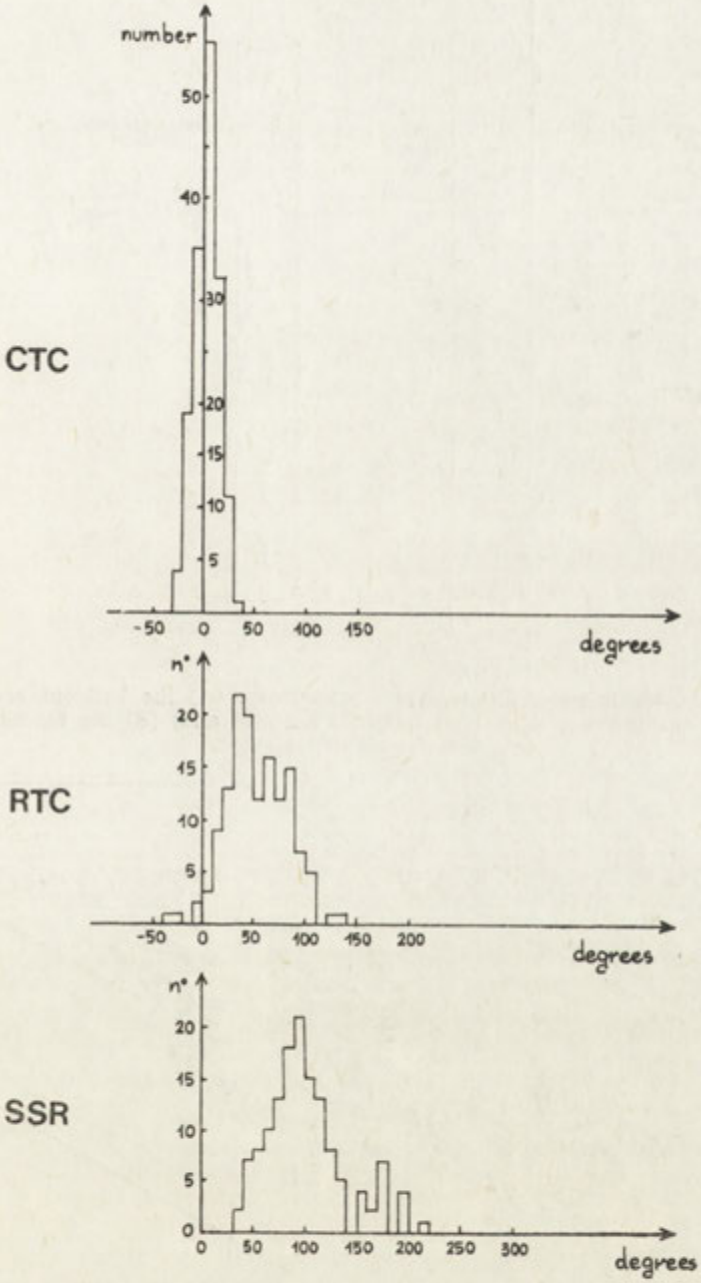

Fig. 7. The distribution of the values of the angles by which a trajectory is changed by CTC (n = 158; $\alpha^\circ = 4^\circ \pm 13^\circ$), RTC (n = 140; $\alpha^\circ = 54^\circ \pm 30^\circ$) and SSR (n = 138; $\alpha^\circ = 101^\circ \pm 37^\circ$)

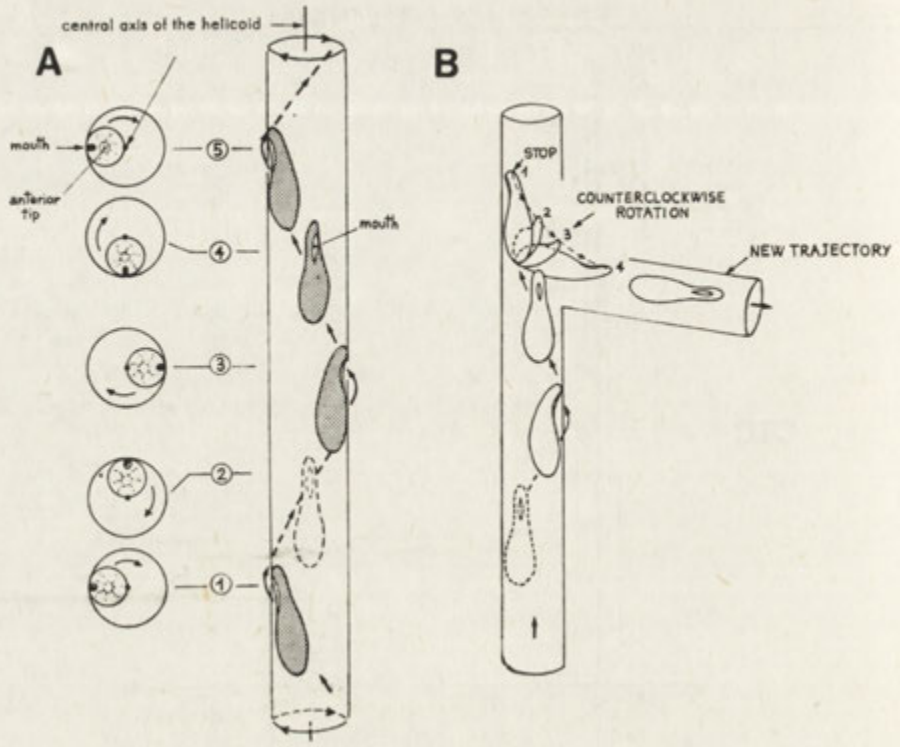

Fig. 8. The swimming of *Blepharisma japonicum*: (A) the helicoid and the positions of a swimming cell as seen from its anterior end; (B) the reaction affecting the change of trajectory

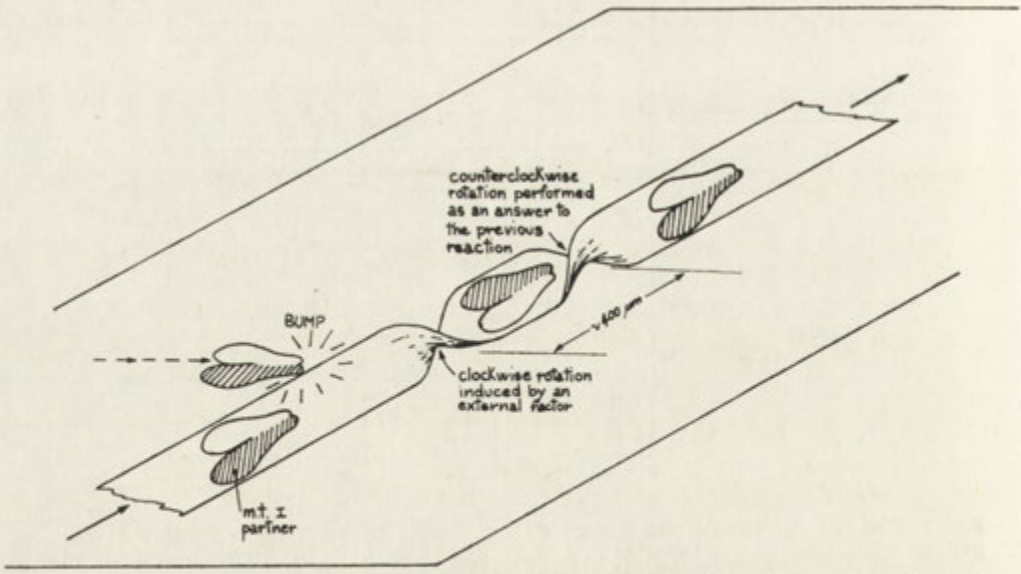

Fig. 9. When the preferential disposition of the heterotypic pair on the substrate (with the mt I partner on the right) is lost (for instance upon bumping against a second pair), it is rapidly restored

208 reactions have been recorded and studied. While the CTC is quite similar to that of the single cells, during the RTC (Fig. 10 A) a pair stops for a short period (1/10 sec) and rotates clockwise around a well-defined point, namely the posterior limit of the cytoplasmic bridge between the conjugating partners.

The SSR is very like the SSR already described for the single blepharisma: the pair stops and immediately rotates clockwise while creeping backwards for ~ 300-400 μm (Fig. 10 B).

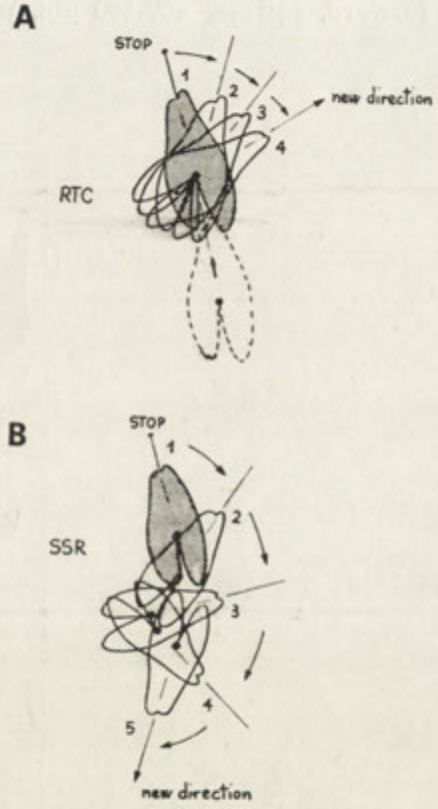

Fig. 10. Homotypic pair: A — The RTC is characterized by a rotation around a point, lying at the level of the posterior limit of the cytoplasmic bridge uniting the partners. B — The SSR is easily recognized by the backward motion of the pair and by the wide clockwise rotation

The CTC changes the trajectory of an angle as wide as $-4.6° \pm 18.7°$ ($n = 77$), while the same angle is $40° \pm 31°$ ($n = 88$) for the RTC and $233° \pm 91°$ ($n = 43$) for the SSR. The velocity of these pairs is, on an average, 132 μm/sec ± 67 μm/sec ($n = 29$): it must be said, however, that such values are somewhat meaningless for the pairs, inasmuch as the instantaneous velocity of a heterotypic pair varies so widely and continuously, that any attempt at describing it only by means of theoretical mean velocity cannot but result in a misleading picture of the true phenomenon.

Swimming

The heterotypic pairs swim at a velocity of 336 μm/sec ± 57 μm/sec along a clockwise helicoid: during this movement, the shorter and lighter partner, belonging to the mt II, is always the external one and the longer and darker partner, belonging to the mt I, is the internal one (Fig. 11). The pitch of the 26 helicoids studies is 1328 μm ± 294 μm and the radius 92 μm ± 16 μm. When the direction of swimming is changed, the pair ceases turning around the central axis of the helicoid and swims forward, shifting 600-800 μm, parallel to itself and turning about 90°

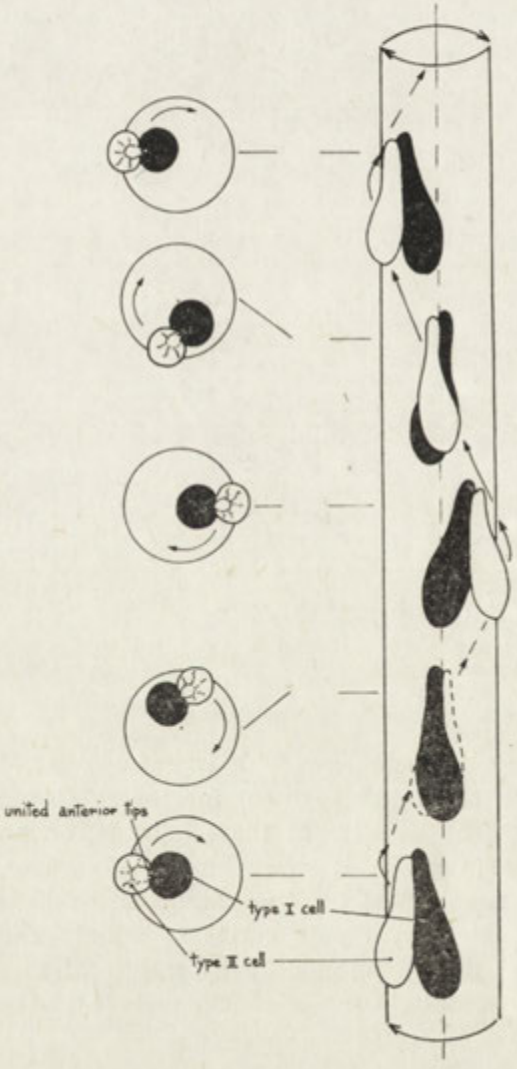

Fig. 11. The clockwise helicoidal swimming a heterotypic pair of *Blepharisma japonicum*

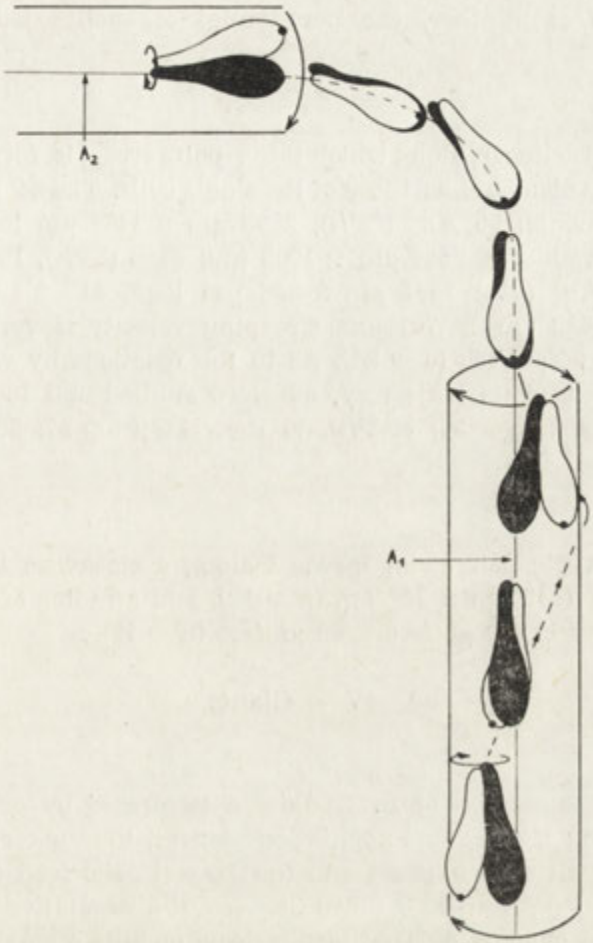

Fig. 12. The reaction affecting the change of direction of the swimming heterotypic pair, from the first direction (A_1) to the second (A_2)

in whichever direction it may be, before resuming its helicoidal swim, along the new axis (Fig. 12).

III — The behaviour of homotypic pairs

Although the homotypic pairs can belong to two different types (namely the I-I type and the II-II type) so far only the I-I type has been studied thoroughly and in this paper, whenever speaking of homotypic pairs, reference is made to this kind (I-I) of homotypic pair. These pairs differ from the heterotypic pairs, mainly because of the absence of any true cytoplasmic bridge: the cell surfaces are juxtaposed and it is only through very narrow "holes" of their membranes that the two cytoplasms

continue into each other, the peristomial organelles being preserved almost intact.

Creeping

The piecewise linear, along which these pairs creep, is formed by the same qualitative elements as those of the single cells. The 24 tracks studied have 104 elements: 65 A+ (62%), 2895 μm ± 1362 μm long, 73° ± 45° wide, with a radius of 2554 μm + 1069 μm; 34 S (33%), 1544 μm ± 1082 μm long; 5 A− (5%) 2094 μm ± 936 μm long, 41° ± 18° wide, with a radius of 2688 μm ± 701 μm. Creeping velocity is very constant at 407 μm/sec ± 94 μm/sec (n = 24). As to the reactions by which a homotypic pair changes its trajectory, 89 were studied and found to belong to the CTC type (n = 34; 38.2%), to the RTC (n = 27; 30.3%) and the SSR (n = 28; 31.4%).

Swimming

The homotypic pair swim forward along a clockwise helicoid which has a pitch of 1412 μm ± 282 μm (n = 12), and a radius of 88 μm ± 17.2 μm. Its velocity is 460 μm/sec ± 60 μm/sec (n = 12).

IV — Giants

Ingestion

A normal population of *B. japonicum* is formed by cells of a fairly uniform length: if the same population starves, then a certain percentage of very small cells appears and the largest cells feed of these small cells, grow in length up to 3 times the size of a vegetative cell: the body shape does not change. A large blepharisma engulfs a small one casually encountered within 30″ — 45″ (Fig. 13 A): as soon as the prey is trapped in the cytostome, the anterior part of the predator bends ventrally, actively pushing the prey into a huge cytoplasmic vacuole.

Creeping

Although 4.5% of segments have been described in the tracks of the giants of *B. japonicum*, the remaining 95.5% actually represents the most relevant percentage of motor patterns and completely belongs to the A− (leftward) type: no A+ (rightward) arc has ever been observed.

Nineteen tracks have been studied (Fig. 13 B) and 173 A− plus 8 S have been described: while the segments are 1410 μm ± 496 μm long, the A− (Fig. 14 A) are 2507 μm ± 1273 μm long, 140° ± 93° wide and have radii ranging around 1178 μm ± 535 μm. The same cell tends to use the same radius, although there may be a wide range of possibilities

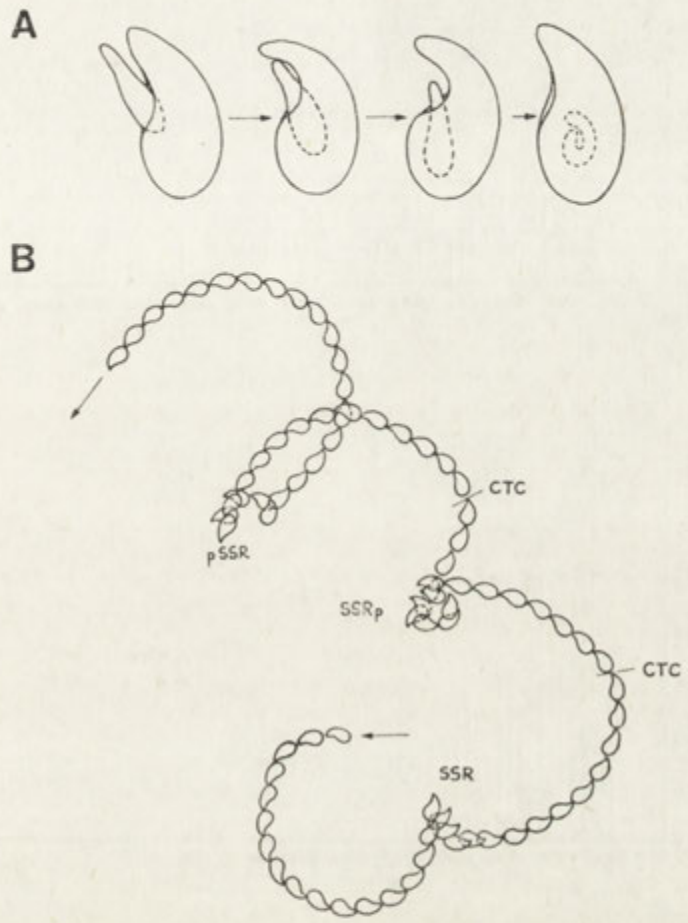

Fig. 13. A — Ingestion of a small *Blepharisma* by one larger form (cannibal). B — the typical track of a cannibal of *Blepharisma japonicum* is formed by a series of leftward arcs (A−), interrupted by the different types of reactions: this scheme has been redrawn from the track n. 3, as recorded from the T. V. screen

for the population as a whole (Fig. 14 B). Four different types of reactions affecting the changes of trajectory have been described: the CTC (n = 85; 46%), the RTC (n = 7; 4%), the SSR (n = 69; 38%), the prolonged SSR (n = 23; 12%). The first three motor patterns are quite similar to those already described for the normal cells and they proved to affect the trajectory by angles which are as wide as $-3° \pm 16°$ for the CTC, as $25° \pm 14°$ for the RTC and as $157° \pm 82°$ for the SSR. The "prolonged SSR" can be described as a backward creeping along a subcircular pathway (the radius ranges around 150 μm) for, at least, two round angles: it lasts about 5″-10″ and the final result (from the point of view of the angle affecting the direction of the creeping) depends upon the

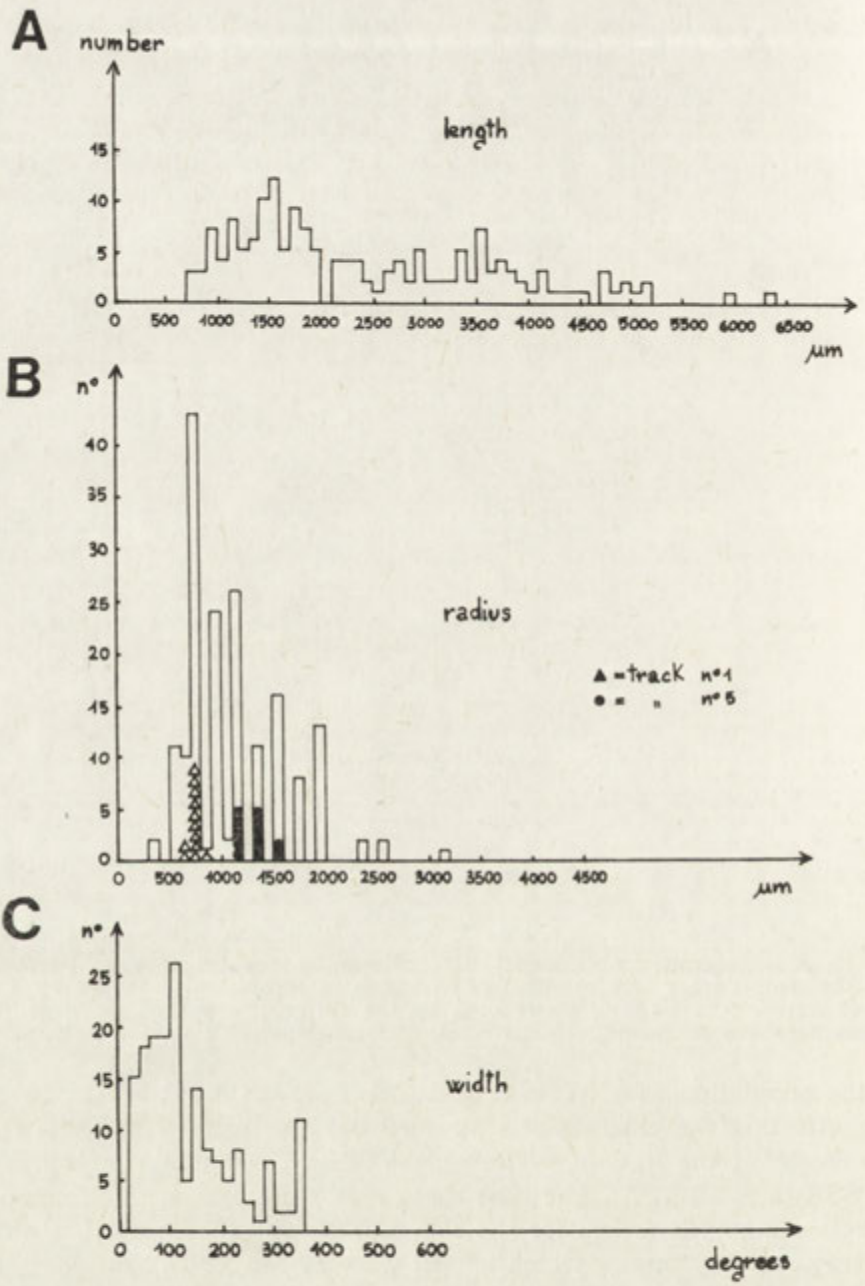

Fig. 14. The distribution of the values of the lengths (A), of the radii (B) and of the widths (C) of the A− observed for the giants of *B. japonicum* are reported; the average length is of 2507 μm + 1273 μm (n = 171), the average radius is of 1178 μm + 535 μm (n = 173); the average width is of 140° + 93° (n = 171)

duration of the whole reaction. The velocity of a cannibal is 198 μm/sec ± 61 μm/sec, far smaller than for the normal cells, very likely due to (1) the frequency and (2) the duration of the interruptions and (3) the prolonged SSR, which are fairly frequent and long-lasting.

Swimming

A giant swims only rarely so that, for our recording sessions, the giants were picked up by a micropipette and then released in the water: they tend to drop downwards, along a helicoid quite difficult to measure and to describe geometrically, due to its rare occurrence and to its downward direction.

Discussion

The behaviour of *B. japonicum*, has been described qualitatively and quantitatively, and its ethogram does not differ radically from those of the species already studied from the same point of view: *O. bifaria* (Ricci 1981), *E. crassus* (Ricci et al. 1987), *L. lamella* (Ricci and Verni 1988) and *Aspidisca* sp. (Ricci et al. in prep.). Such a conclusion is indicated by the helicoidal swimming and by the creeping tracks which result from the combination of the same geometric elements, namely A−, S and A+, and of the same motor reactions (CTC, RTC, SSR). This seems to suggest a functional basis shared by all of these five species, at least as far as the basic elements responsible for their motor capabilities are concerned: however, it must be remembered that specific investigations of the fundamental physiological bases of motility of different ciliates (i.e. functions of membranelles, cirri, cilia under defined conditions, etc.) are required in order to draw any conclusions in this field.

The double nature of populations of *B. japonicum* represents a peculiarity distinguishing this ciliate from the other four: 35% of the cells creep only along segments or leftward arcs, while the remaining 65% creep only along segments or rightward arcs: *Oxytricha* creeps only along leftward arcs (Ricci 1981), *Litonotus* only along rightward arcs (Ricci and Verni 1988) and both *Euplotes* (Ricci et al. 1987) and *Aspidisca* (Ricci et al. in prep.) can follow leftward arcs, segments and rightward arcs without any restriction. Taking into consideration the facts that *Blepharisma* creeps on the substrate with the adoral membranelles to the left (cf. Fig. 1) and that no apparent morphological difference occurs between the two populations of *B. japonicum*, it seems possible to conclude that such behavioural difference might be due rather to functio-

nal (different ciliary beating; different ways of synchronizing the beat etc.) rather than to morphological diversities. The clockwise rotation occurring in all of the *Blepharisma* performing an SSR seems to strengthen such a hypothesis.

The noticeable constancy of the quantitative parameters describing the ethogram of *B. japonicum* (namely their small standard deviations) distinguishes this species from *Oxytricha*, *Euplotes* and *Aspidisca*, which, on the contrary, behave in accordance with very widely changing motor patterns. On the other hand, it must be remembered that each single ciliate (regardless of the species it belongs to) tends to move along tracks very constant in their quantitative aspects (length and width of the arches, velocities, length of the radii, as shown in Fig. 3 B for *Blepharisma*), thus showing a certain degree of individuality as far as behaviour is concerned. According to this consideration, the standard deviations observed for the quantitative parameters of certain population are a measure of its more or less heterogenous composition, and consequently *B. japonicum* is a species formed by approximately homogeneous individuals, while the populations of the three Hypotrichs are formed by cells behaving differently from each other. The possible adaptive significances of the two population structures (uniform vs heterogeneous) are at present being investigated in our Laboratory. Different states of *B. japonicum* move at different velocities: single cells, 279 μm/sec ± 72 μm/sec; heterotypic pairs, 132 μm/sec ± 67 μm/sec; homotypic pairs, 339 μm/sec ± 94 μm/sec; cannibals, 198 μm/sec ± 61 μm/sec. The heterotypic pairs are the most irregular from this point of view. According to what is known about electrophysiology and locomotion in ciliates (Naitoh 1982, Machemer 1986, Machemer and Deitmer 1987) it seems appropriate to conclude that the heterotypic pair of *B. japonicum* is quite unbalanced and unstable from this point of view, although the physiological basis and the adaptive significance of such a phenomenon are still far from being accounted for.

If we now consider the velocities, expressed in relation to the length of the different states, the above-mentioned values become 0.25 RU/sec for the cannibals, 0.4 RU/sec for the heterotypic pairs, 0.88 RU/sec for the single cells, 1.1 RU/sec for the homotypic pairs. While the difference between normal cells and homotypic pairs is somewhat subtle, the velocities of heterotypic pairs and of giants deserve more specific analysis. The striking inertia of the giants is very likely due to their cross-section, which is about four times as large as that of the normal cells (Giese 1973): in accordance with this datum, the velocity cannot but drop to the observed values, being the dramatically increased resistance of the water only poorly compensated for by the relatively slight increase

of the somatic and perioral ciliature. The reduced velocity of the heterotypic pairs, on the contrary, seems to be a consequence of the loss of the peristomial ciliary organelles characterizing this kind of pair: if this is true, the role of the adoral ciliature in contributing of the general velocity of this species would be demonstrated, the somatic ciliature being approximately the same as in normal cells.

The creeping pathway of a heterotypic pair is characterized by frequent (67%) leftward arcs: this behavioural trait might be interpreted as the consequence of the asymmetric morphology of the heterotypic pair itself, in which the m.t. I-partner (360 μm long) lies on the right of the m.t. II-partner (300 μm long): the larger cell would work as a more powerful engine than the left partner, thus inducing the pair to creep along leftward arcs.

The different kinds of reactions affecting the changes of trajectory in *B. japonicum*, namely the CTC, the RTC and the SSR, are performed with about the same frequency (36%, 32%, 32%, respectively) and result in changes of the trajectories having angles as wide as 4°, 40° and 150°, respectively. This picture, together with the geometry of these reactions (cf. Figs. 4, 5, 6), seems to indicate that CTC, RTC and SSR are likely to belong to the same basic kind of reaction, differing from each other only in respect of the quantitative effects on the track. On the contrary, the reason why *B. japonicum* never performs the STC, a reaction frequently observed in the other four species already studied, appears to be more puzzling. Among them, *B. japonicum* is the only species with a rounded body which contacts the substrate only along a rather narrow strip of cell surface, while *L. lamella* and the three Hypotrichs present a wide ventral surface. It may be presumed that an STC is performed by the three Hypotrichs (and by *L. lamella*), differentially changing the beating parameters and/or the thigmotactic forces of the right and left cirri (cilia) (cf. Fenchel 1987): the STC would resemble the maneuvre of a tractor, which is capable of expressing specifically and selectively different forces at the right or at the left side of its structure.

As to the frequencies of the geometrical elements (A−, S, A+) which follow another A−, S, A+ respectively (cf. Fig. 2 B-D), it is evident that there is a strong tendency for *B. japonicum* to repeat one certain element (contrary to what happens for *Aspidisca* sp.), although it is quite difficult to find any physiological explanation for such peculiar behaviour, at least on the basis of present-day knowledge.

Comparison of different ethograms shows that the creeping velocities of *Blepharisma, Litonotus, Oxytricha, Euplotes, Aspidisca*, are 0.3, 0,75, 4, 9 and 7 RU/sec respectively. These data seem to separate *Litonotus* and *Blepharisma* from the three Hypotrichs, in which both the body

shape and the ciliary organelles are far more differentiated and sophisticated. The same is also true in respect of their swimming velocities: 1.5 RU/sec for *Blepharisma*, 1 RU/sec for *Litonotus*, 7 RU/sec for *Oxytricha*, 8.5 RU/sec for *Euplotes*, no significant value being found for *Aspidisca*, a species definitely incapable of active swimming. As a general conclusion to this paragraph we may say that the species so far studied for their motility creep forward fairly constantly on the substrate along segments (uniform rectilinear motion = "cellular engines balanced") and arcs (uniform circular motion = "cellular engines uniformly unbalanced") and that the successive traits are joined to each other by the same kinds of reactions. Far from representing a mere naturalistic oddity, this conclusion might indicate that the basic mechanisms controlling the motor behaviour of the different species are very similar to each other in the different species, and that the differences among them are mainly represented by quantitative variations of the same qualitative parameters, the only exception so far individuated being the lack of the STC in *B. japonicum*.

As to the way of performing an SSR, the *L. lamella-B. japonicum* group significantly differs from that of the Hypotrichs: indeed, while the first two creep backwards and turn clockwise at the same time, the other three clearly separate the two components, turning clockwise only at the end of the backward motion. Whether such a clearcut difference in the manoeuvering capacities occurring between the *L. lamella-B. japonicum* group and the *O. bifaria-E. crassus-Aspidisca* sp. group also mirrors a phylogenetic difference among them (Corliss 1979) or, rather, only opportunistic convergences (Small and Lynn 1986) remains to be ascertained and further evidence is required in order to be able to draw any conclusion in this sense.

As suggested by Nanney (1980, 1986), the study of the behaviour of *Protozoa* will result into a better and more general understanding of their biology: the ethograms so far drawn for ciliates seem not only to support this line of thought, but also to justify the efforts made to make the ethogram more precise and reliable (Russo et al. 1988).

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REFERENCES

Corliss J. O. 1979: The ciliated *Protozoa*. Characterization, classification and guide to the literature. 2nd ed. Pergamon Press, Oxford.

Eibl-Eibesfeldt I. 1967: Grundriss der vergleichenden Verhaltensforschung Ethologie. R. Piper and Co. Verlag, München.
Fenchel T. 1987: Ecology of *Protozoa*. The biology of free-living phagotrophic Protists. Springer, Berlin-Heidelberg-New York.
Giese A. C. 1946: An intracellular photodynamic sensitizer in *Blepharisma*. J. Cell. Comp. Physiol., 28, 119-127.
Giese A. C. 1973: *Blepharisma*. The biology of a light-sensitive protozoan. Stanford Univ. Press, Stanford (California).
Jennings H. S. 1906: Behaviour of lower organisms. Indiana University Press, Bloomington, London, 2nd ed. 1976.
Kung C. 1976: Foreword to the 1976 Edition. In: Behaviour of the lower organisms, H. S. Jennings, Indiana University Press, Bloomington, London; 2nd ed. IX-XI.
Machemer H. 1986: Electromotor coupling in cilia. Fortsch. Zool., 33, 205-250.
Machemer H. and Deitmer J. W. 1987: From structure to behaviour *Stylonychia* as a model system for cellular physiology. Progr. in Protistol., 2, 213-330.
Miyake A. 1968: Induction of conjugation by cell-free fluid in the ciliate *Blepharisma*. Proc. Jpn. Acad., 44, 837-841.
Miyake A. 1978: Cell communication, cell union, and initiation of meiosis in ciliate conjugation. Curr. Top. Dev. Biol., 12, 37-82.
Naitoh Y. 1982: *Protozoa*. In: Electrical conduction and behaviour in "simple" Invertebrates (Ed. G. A. B. Shelton), Clarendon Press, Oxford, 1-48.
Nanney D. L. 1980: Experimental ciliatology. J. Wiley and Sons, New York.
Nanney D. L. 1986: Introduction. In: The molecular biology of Ciliated *Protozoa* (Ed. J. G. Gall), Academic Press Inc., Orlando, Florida, U.S.A.
Raab O. 1900: Ueber die wirkung fluorescierender Stoffer auf Infusorie. Zeitschr. Biol., 39, 534-546.
Ricci N. 1981: The ethogram of *Oxytricha bifaria* Stokes (*Ciliata, Hypotrichida*). I. The motile behaviour. Acta Protozool., 20, 393-410.
Ricci N. 1982: The ethogram of *Oxytricha bifaria* Stokes (*Ciliata, Hypotrichida*). II. The mating behaviour. Acta Protozool., 21, 13-23.
Ricci N. 1986: The ethological approach to the motility of *Protozoa*: a new attitude toward an old problem. In: Biology of Reproduction and Cell Motility in Plants and Animals (Eds. M. Cresti and R. Dallai), University of Siena, Siena, 211-216.
Ricci N. and Miceli C. 1983: Ethograms of Ciliates. Proceedings of the 5th European Conference on Ciliate Biology, Geneva, Sept. 5-9.
Ricci N., D'Arrigo, M., Bracchi P. and Dini F. The ethogram of *Aspidisca* sp. (in prep.).
Ricci N., Giannetti R. and Miceli C. 1987. The ethogram of *Euplotes crassus* (*Ciliata, Hypotrichida*). I. The wild type. Eur. J. Protistol., 23, 129-140.
Ricci N. and Verni F. 1988: Motor and predatory behavior of *Litonotus lamella* (*Protozoa, Ciliata*). Can. J. Zool., 66, 456-465.
Russo A., Gualtieri P. and Ricci N. 1988: A semi—automatic computerized analysis of tracks of Ciliates. Experientia, 8, 277-280.
Small E. E. and Lynn D. H. 1985: Phylum Ciliophora Doflein, 1901. In: An illustrated guide to the Protozoa (Eds. J. J. Lee, S. H. Hutner and E. C. Bovee), Allan Press, Lawrence, Kansas, 393-575.
Van Houten J., Hauser D. C. R. and Levandowsky M. 1981: Chemosensory behaviour in *Protozoa*. In: Biochemistry and Physiology of *Protozoa* (Eds. M. Levandowsky, S. H. Hutner), Academic Press, New York, vol. 4, 67-124.

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Cytoplasmic Zones Responsible for Differentiation of the New Macronucleus and Degradation of the Old in *Chilodonella steini* (*Trithigmostoma steini*)

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Synopsis. As a result of division of the synkaryon in *Chilodonella steini* two nuclei are produced, one of them becomes the new macronucleus and the other the micronucleus. Unlike *Tetrahymena, Paramecium* and *Blepharisma*, no precisely defined cell zones responsible for the determination of this differentiation have been found in the case of *Chilodonella*.

Initially the micronucleus, macronuclear anlagen, and the old macronucleus are located in the centre of the cell, mainly on the left side. As the macronuclear anlagen grows, the location of nuclei in the cell changes. Micronucleus and macronuclear anlagen shift to the front, under the oral apparatus, while the old macronucleus moves to the posterior part of the cell, where it is degraded. When the old macronucleus has been completely disintegrated, the micronucleus and macronuclear anlagen return to their central position in the cell.

Polarity of the oocyte cytoplasm plays an important role in cell differentiation in many eukaryotic organisms (Tobler 1973, Beermann and Meyer 1980, Matuszewski 1982). It is belived that cell differentiation is caused by certain determinants located in special zones of the cytoplasm. The character of these determinants, however, is to a great extent unknown.

In ciliates at least two stages of conjugation are known, in which the fate of the nuclei depends on their location in the cell. The first

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stage is connected with the retention or degeneration of nuclei which are produced during the micronucleus meiosis (Radzikowski 1972, Yanagi and Hiwatashi 1985, Mikami 1985, 1987). The second stage is connected with the differentiation of the new micronucleus and macronucleus (Nanney 1953, Grandchamp and Beisson 1981, Mikami and Ng 1983, Mikami 1985, 1987, Weiske-Benner and Eckert 1987).

Simultaneously with the process of differentiation and development of the new nuclear apparatus, the process of degeneration of the old macronucleus takes place. The changes connected with this take a similar course in various ciliates, e.g., in *Euplotes* (Raikov 1982, Fidler and Kloetzel 1984), *Paramecium caudatum* (Mikami 1979), or *Tetrahymena* (Simon and Doerder 1981). All authors agree that there are zones of cytoplasm which are responsible for the degradation of the old macronucleus in these species.

The existence of cytoplasmic zones determining the differentiation of the new nuclear apparatus has been established for ciliates belonging to *Oligohymenophora*, the cells of which are cylinder or pear-shaped. However, even in these animals the locations of zones in cells are different and while nucleus differentiation into a new macronucleus occurs in the anterior part of the cell in *Tetrahymena*, in *Paramecium* it takes place in the posterior part. Thus it seems that the shape and spatial organization of the intercellular structures may play an important role in these processes.

It therefore seemed interesting to study this issue for *Chilodonella steini*, a ciliate belonging to *Kinetofragminophora*, whose cell is strongly dorsoventrally flattened and the oral apparatus shifted to the ventral side.

In *Chilodonella* a typical conjugation is observed, as a result of which a new nuclear apparatus is formed and the old macronucleus is degraded. There are no data, however, as to whether the fact that the nucleus occupies a certain position in the cell has an influence on its differentiation.

The aim of our research was thus to establish whether separate cytoplasmic zones exists responisible for the differentiation of the micronucleus and macronuclear anlagen, and whether there is a specific zone in the cell in which degradation of the old macronucleus takes place.

Material and Methods

The study was conducted on the ciliate *Chilodonella steini* (Radzikowski and Gołembiewska 1977) belonging to the class *Kinetofragminophora*, subclass *Gymnostomata*. *Chilodonella steini* is a ciliate dorsoventrally flattened, with

the oral apparatus and almost the whole ciliature located at the ventral side of the cell.

The nuclear apparatus consists of the micronucleus and macronucleus of the heterometric type.

Research was carried out on the 10-87 strain at the period of its maturity for conjugation.

The ciliate was cultivated according to the method described by Radzikowski and Gołembiewska (1977), Radzikowski (1985). Conjugation was produced according to the method described by Kaczanowski et al. (1980). The experiments were carried out on pairs of cells in the course of conjugation. The cells were cultured in culture dishes until the pairs separated and then the exconjugant cells were fixed 6, 14, 48 and 60 hours after the moment of cell separation of the conjugants. The control group was composed of vegetative cells of this strain, fixed 6 hours after the division. At that time the zones of macronucleus develop and both the nuclei occupy their typical positions in the central part of the cell (Radzikowski 1985).

The ciliates were fixed in a mixture of alcohol and acetic acid (3:1) and then stained with the Feulgen method, while the cytoplasm was stained with light green. The stained nuclei and cells were outlined under the microscope with the outlining apparatus. The locations of nuclei in the respective hours of the experiment were then marked on a sketch of an "average cell" for each hour of experiment. In this way sets of points (a point corresponds to the centre of a nucleus), corresponding to the locations of respective types of nuclei at respective hours of postconjugation reorganization were obtained. To define the frequency of occurrence of a given type of nucleus in a cell, the cell was arbitrarily divided transversally into six zones: I and II located above the oral apparatus, and III, IV, V and VI located below it. Each zone was divided into two parts, the right and the left, by a line passing through the oral apparatus (Fig. 1). Then respective types of

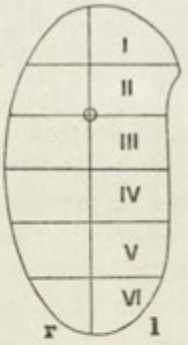

Fig. 1. Diagram of arbitrary division of the cell into zones. r — right side, l — left side, I-VI — zones of the cell

nuclei in each zone were counted, indicating whether they were on the right or left side. Sides of the cell, right and left, are indicated according to desmodexy rule (Radzikowski and Gołembiewska 1977, Corliss 1979, Hoffmann 1986). All figures present cells from their ventral side.

The following abbreviations were used:
Mi — micronucleus
Ma — macronucleus
Maz — anlagen of a new macronucleus
Nu — undifferentiated nuclei

Results

The frequency of occurrence of various nuclei in respective cell zones during postconjugational reorganization and in control cells is presented in Table 1 a-f.

The location of nuclei in the cell in respective hours has been graphically presented in Figures 2, 3.

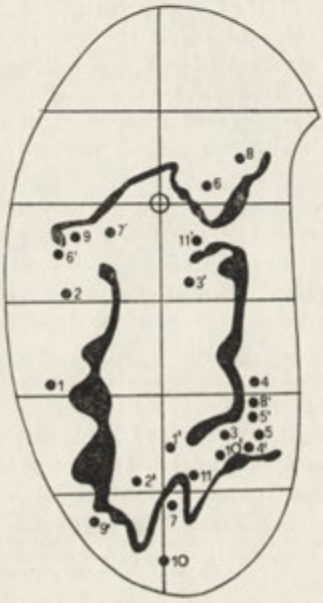

Fig. 2. Location the Mi and the Maz in exconjugant cells 6 hours after the separation. The same figures mean sister nuclei, 1-11, 1'-11' Maz. Dark areas represent regions where undifferentiated nuclei are present

Micronucleus location in a vegetative cell

In control vegetative cells, six hours after the division, the Mi occupies the central position, most often in zone IV on the right and left side of the cell, respectively in 37 and 34 per cent of cases. In 60 per cent of all cases, the Mi is located on the right side of the cell. It never occurs in zones I and II above the oral apparatus, but sporadically occurs in zone VI of the cell (one case) (Tab. 1 a).

Micronucleus location during the postconjugational reorganization of the nuclear apparatus

Analysis has revealed that six hours after separation of cells the Mi and the early Maz were located randomly in the cell, e.g., nuclei 1 and 1', 2 and 2', 3 and 3', 4 and 4' etc. (Fig. 2).

It has been found that both Mi and Maz occur in the same cell zones. At that hour undifferentiated nuclei (Nu) are most frequent. These nu-

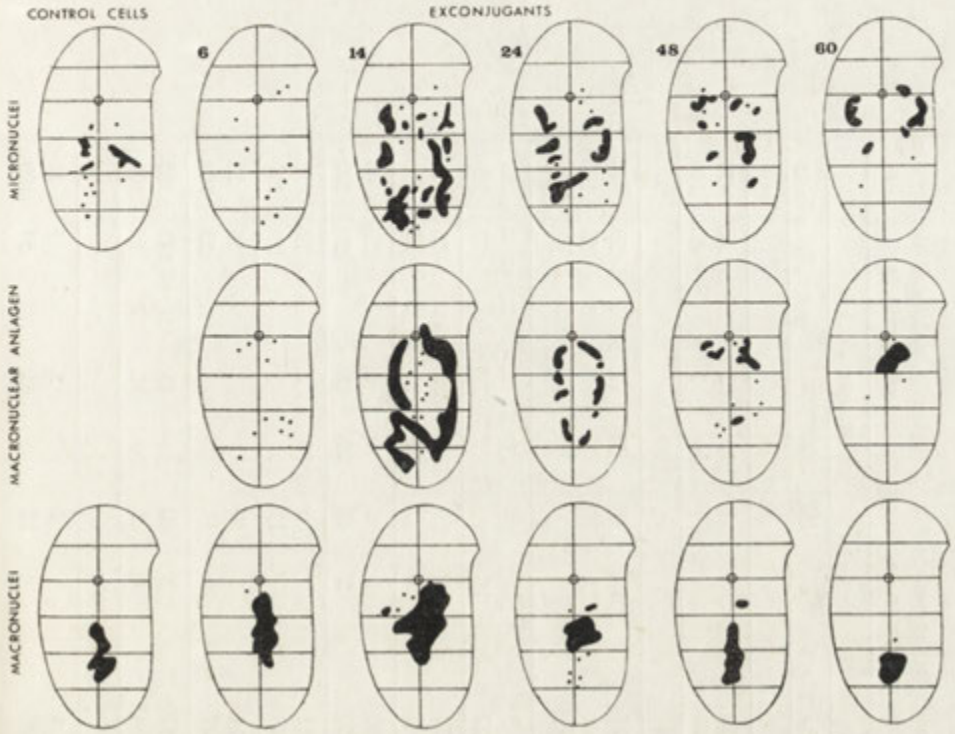

Fig. 3. Location of Mi, Ma and Maz in respective cell zones in vegetative and exconjugant cells in respective hours of the nuclear apparatus reorganization. The dark areas represent regions where the respective kinds of nuclei were present

clei do not form any specific aggregation but occur in all, except the I, zones of the cell. They are more often located on its left side (59 per cent of cases) (Tab. 1 b, Fig. 2 and 3).

In exconjugant cells, in later periods of reorganization in the 14th and 24th hour a gradual decrease of the Mi number in zones V and VI and their central aggregations in zones III and IV have been observed. The fraquency of occurrence of Mi in these zones is presented in Tab. 1 c.

In exconjugant cells, in the 48th and 60th hour after separation the Mi occurs in 80 per cent of cases in zones III and IV, and sometimes in zone II (Tab. 1 c). In control cells, however, the occurrence of Mi in zone II has never been observed (Fig. 3).

Location of the macronuclear anlagen in exconjugant cells

In exconjugant cells, 6 hours after cell separation, Maz, like Mi, occur in zones III and V in one case in zone VI (Tab. 1 d).

In the 14th hour Maz can be mainly observed in zones III, IV, V and sporadically in zones II and VI (Tab. 1 d). Maz occur more medially

Table 1

Frequencies of occurrence of various types of nuclei in respective zones of a *Chilodonella steini* cell during the postconjugational reorganization of the nuclear apparatus and in vegetative cells (control)

h		right side							left side							
		I	II	III	IV	V	VI	E_r	I	II	III	IV	V	VI	E_l	E_{r+1}
a) micronuclei in vegetative (control) cells																
	N			1	11	5	1	18			1	10	1		12	30
	%			3	37	17	3	60			3	34	3		40	100
b) non-differentiated nuclei in exconjugant cells																
	N		1	7	9	6	4	27		10	11	5	12	1	39	66
	%		1	11	14	9	6	41		15	17	8	18	1	59	100
c) micronuclei in exconjugant cells																
6	N			1	2			3		2		1	3	2	8	11
	%			9	18			27		18		9	28	18	73	100
14	N			15	15	21	18	69		1	19	22	32	9	83	152
	%			10	10	14	11	45		1	13	14	21	6	55	100
24	N			5	7	7	1	20		1	5	8	6		20	40
	%			12	17	17	2	50		2	12	20	15		50	100
48	N		1	8	3	1		13			5	10	2		17	30
	%		3	27	10	3		43			17	33	7		57	100
60	N			10	2	1	1	14		3	10	3			16	30
	%			34	7	3	3	47		10	33	10			53	100
E	N		1	39	29	30	20	120		7	40	44	43	11	145	265
	%		1	32	24	25	17	45		4	28	30	30	8	55	100
d) macronuclear anlagen in exconjugant cells																
6	N			2		1	1	4			2		5		7	11
	%			18		9	9	36			18		45		64	100

14	N	17	8	23	6	54	4	31	25	33	5	98	152
	%	11	5	15	5	36	3	20	16	22	3	64	100
24	N	7	7	3		17		8	8	7		23	40
	%	18	18	7		42		20	20	18		58	100
48	N	5		3		8		16	3	3		22	30
	%	17		10		27		53	10	10		73	100
60	N	10	1			11		18	1			19	30
	%	33	4			37		60	3			63	100
E	N	41	16	30	7	94	4	75	37	48	5	169	263
	%	16	6	11	3	36	1	29	14	18	2	64	100
e) macronuclei in vegetative (control) cells													
	N		6	7		13			7	10		17	30
	%		20	23		43			23	34		57	100
f) old macronuclei in exconjugant cells													
6	N	3	6			9		9	33	5		47	56
	%	5	11			16		16	59	9		84	100
14	N	10	41	2		53		21	73	5		99	152
	%	7	27	1		35		14	48	3		65	100
24	N	1	9	2		12		3	21	4		28	40
	%	2	23	5		30		7	53	10		70	100
48	N		5	7		12		3	4	11		18	30
	%		17	23		40		10	13	37		60	100
60	N			11		11			1	15		16	27
	%			41		41			4	55		59	100
E	N	14	61	22		97		36	132	40		208	305
	%	15	62	23		32		17	63	19		68	100

Abbreviations: h – hours, N – number, E_r – the sum of nuclei on the right side of the cell, E_l – the sum of nuclei on the left side of the cell, I–VI – zones of the cell, 6–60 – hours after separation

with respect to Mi, forming a pronounced ring close to the centre. Sporadically, the occur in the very centre (Fig. 3). A ring-shaped, but less compact Maz distribution can be observed in cells after 24 hours. At that time Maz cannot be observed in zones II and VI (Tab. 1 d, Fig. 3).

In the 48th hour Maz occur mainly in zone III (70 per cent of cases) and less frequently in zones IV and V (30 per cent of cases).

In the 60th hour after cell separation Maz occur mainly in zone III (93 per cent of cases), and more often on the left side of the cell (63 per cent of cases) (Tab. 1 d, Figs, 4 A and B).

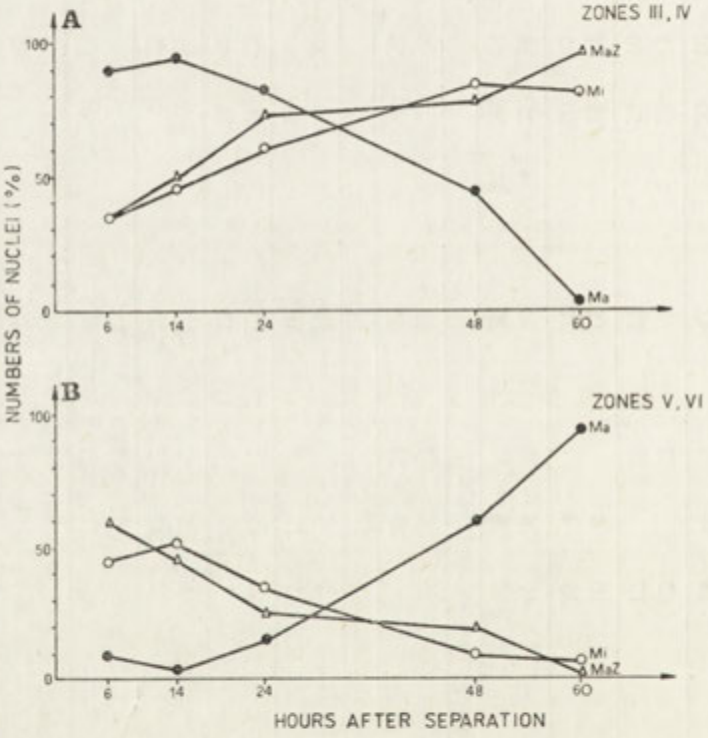

Fig. 4. Changes of the frequency of occurrence of various nuclei types during the reorganization of the nuclear apparatus in the zones III and IV (A) and V and VI (B)

Macronucleus location in vegetative cells

In vegetative cells, the Ma is located in their central part. Its occurrence is limited to zones IV and V and it occurs on the left side in 57 per cent cases (Tab. 1 e, Fig. 3).

Location of the old macronucleus in respective hours of postconjugational reorganization

In exconjugant cells, 6 hours after cell separation, the Ma occupies the medial position in the cell zones III, IV and V. In 84 per cent of cases this nucleus occurs on the left side of the cell. Such a position can be observed also after the 14th and 24th hour. Only the frequency of occurrence on the right or left side of the cell changes, yet the left side location prevails (14th hour in 65 per cent and 24th hour in 70 per cent of cases) (Tab. 1 f, Fig. 3).

In the 48th hour, the Ma occurs most frequently in zone V of the cell (65.5 per cent of cases) and less numerously, it occurs in zones III and IV on the left side (23 per cent of cases), while it has not been found in zone III on the right side.

In the 60th hour, in 96 per cent of cases, Ma occurs in zone V and only in 4 per cent, in zone IV (Tab. 1 f, Fig. 3).

Discussion

It seems that the location of nuclei in a *Chilodonella steini* cell is connected with its structure and, in particular, with the location of the basket and food vacuoles (Sołtyńska 1971, Hofmann 1986).

Since the basket and food vacuoles occupy the right side of the cell the majority of all types of nuclei can be found on its left side.

Lack of nuclei of their minimal number in the anterior part is caused by the flattening of the anterior pole of the cell (Radzikowski and Gołembiewska 1977, Hofmann 1986). As a result of a specific arrangement of kinetes in this area the whole interior of the circumoral part of the cell is filled with cortex structures. This may considerably hinder the passage of the nuclei to this area.

Total lack of Ma and minimal fraquency of occurrence of the remaining types of nuclei in the posterior part of the cell (zone VI) are in turn caused by the fact that this zone is occupied by cytopyge and excretion vacuoles.

First Maz occurs in the cell 6 hours after the moment of cell separation of the conjugants. No separate cytoplasm zones responsible for differentiation of either Mi or Maz have been found. This distinguishes *Chilodonella* from *Tetrahymena* (Nanney 1953, Gaertig 1988 pers. com.), *Paramecium* (Grandchamp and Beisson 1981, Mikami and Ng 1983, Mikami 1985, 1987), or *Blepharisma* (Miyake 1988 pers. com.).

All these authors have come to the conclusion that differentiation of nuclei which have originated as a result of the synkaryon division is closely connected with their location in a definite part of the exconjugant cell.

The research conducted by Mikami and Ng (1983) has proved that the removal of Ma from early *Paramecium caudatum* exconjugant leads to aberrations in nuclear differentiation, which are probably connected with disappearance of characteristic cytoplasm zones. This experiment indicates that the old Ma is responsible for activating cytoplasm factors connected with differentiation of the nuclei. This phenomenon was studied in *Tetrahymena* (Nanney 1953), *Paramecium* (Grandchamp and Beisson 1981, Mikami 1985, 1987) and *Blepharisma* (Miyake 1988, pers. com.). In the two former cases nuclei are actively displaced into the zones responsible for their differentiation. Detailed investigations by Grandchamp and Beisson (1981), Mikami (1985), Gaertig (1988 pers. com.), indicate that the factor responsible for nuclei shift is the cytoskeleton of the cell.

Nanney (1953, 1980) observed in *Tetrahymena* displacement of nuclei into opposite poles of the cell through very long, meridionally oriented spindles, which have originated during the division of the synkaryon. Nuclei located in the anterior part of the cell differentiate in Maz, while nuclei in the posterior part, in Mi.

On the basis of these observations, Nanney supposes that differentiation in *Tetrahymena* can be described as a sequence of nucleo-cytoplasmatic interactions.

In Paramecium, Mi and Maz differenteiation is also connected with their location in the cell. Research by Grandchamp and Beisson (1981) and by Mikami (1983, 1985, 1987) has revealed that nuclei located in the anterior part of the cell become Mi and nuclei located in the posterior part, Ma.

In *Paramecium* the anterior-posterior location of nuclei after the last synkaryon division is also a result of a specific function of the division spindle which reaches to the opposite cell poles, and is also connected with a temporary shortening of the cell (Grandchamp and Beisson 1981, Mikami 1985). Location of nuclei in poles is short-termed and after that period the differentiated nuclei shift to the centre.

In *Paramecium* cells in which the posterior part has been cut off Maz do not develop (Grandchamp and Beisson 1981).

The presented data suggested that differentiation of nuclei in Mi and Ma is controlled by cytoplasmic factors.

The question arises as to why the process of Ma differentiation in *Chilodonella steini* is not connected with definite cell zones (Fig. 2).

Perhaps the causes of this phenomenon ought to be sought in the specific structure of the cell of this ciliate (dorsoventral flattening).

According to Corliss (1979), the ancestral form of a ciliate cell was spheroid. At one pole there was the oral apparatus (the anterior part of the cell). The ancestral spheroid form, however, underwent modifications. They consisted mainly in changes of the shape and the resulting shifts of the oral apparatus and cytopyge. As a result new forms originated, i.a., such as *Tetrahymena* and *Paramecium*. Modification of the original shape in the case of these ciliates did not cause changes in cell structure on the anterior-posterior axis.

However, in the case of *Chilodonella steini* the changes of the original form led to extreme flattening of the cell. Perhaps the ventral side with the oral apparatus corresponds to the former front of the hypothetical original cell and the dorsal, to its back. Thus, when observing a *Chilodonella* cell under a microscope we simultaneously see the overlapping back and front end. Such an arrangement results in overlapping of all nuclei, which gives the impression of random diistribution. If we made a similar observation of a *Tetrahymena* or *Paramecium* cell, i.e., from the top, then we should obtain images very similar to those observed for *Chilodonella steini* (Fig. 5).

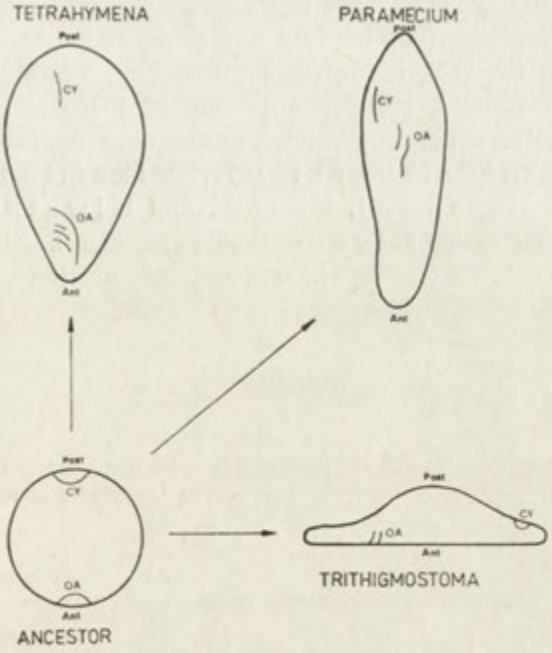

Fig. 5. Hypothetical trends of changes of cell forms in ciliates: *Tetrahymena*, *Paramecium*, *Chilodonella* species. Ant — anterior, Post — posterior, OA — oral apparatus, CY — cytopyge

In the first hours after cell separation the nuclei are located in central zones of the cells. From the 24th hour the shift begins of Mi and Maz to zone III and of old Ma to zone V (Fig. 4). Total spatial separation of respective types of nuclei can be observed by the 60th hour. At that time, the process of degeneration of the old Ma occurs in zone V. It seems that the shift of Mi and Maz to zone III is connected with the differentiation of cytoplasm. Cytoplasm in zone III may at that time contain agents protecting Mi and Maz against degeneration, while cytoplasm in zone V contains factors degrading the old Ma. Mikami and Ng (1983) experimentally removed Maz in *Paramecium tetraurelia*. This led to retention of old Ma. The experiment indicates that production of a specific degradation zone depends on Maz. This is probably connected with Maz reaching an appropriate stage of development. Only when it is transcriptionally active are appropriate autolytic proteins synthesized (Nanney 1980), as a result of which the degeneration zones are produced for the old Ma. The existence of such zones in *Tetrahymena* has been reported by Nanney (1953, 1980), Simon and Doerder (1981).

After complete degradation of the old Ma, Mi and Maz occupy the central position in the cell, as in the vegetative cell, which is probably connected with the disappearance of the deganeration zone.

A similar mechanism of haploid nuclei degradation, connected with their location in the cell, occurs in *Paramecium, Tetrahymena, Blepharisma, and Chilodonella*. Usually only one of them is retained, and it occupies a definite zone. The nuclei undergoing degradation are located outside it (Radzikowski 1972, Mikami 1980, Grandchamp and Beisson 1981, Yanagi and Hiwatashi 1985, Gaertig 1988 pers. com., Miyake 1988 pers. com.). All these authors indicate cytoplasmic factors as causing the degradation of nuclei.

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REFERENCES

Beermann S. and Meyer G. 1980: Chromatin rings as products of chromatin diminution in Cyclops. Chromosoma, 77, 277-283.
Corliss J. O. 1979: The Ciliated *Protozoa*. Pergamon Press.

Fidler S. J. and Kloetzel J. A. 1984: Roles of old macronuclear fragments in nuclear and cortical development following conjugation in *Euplotes*. J. Protozool., 31, 11 A.

Grandchamp S. and Beisson J. 1981: Positional control of nuclear differentiation in *Paramecium*. Dev. Biol., 81, 336-341.

Hofmann A. 1986: Die Stomatogenese der Ciliaten *Chilodonella steini*. Die Merkmale der Kinetosomenvermehrung als Beitrag zur einer phylogenetisch begrundeten Systematik. Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften der Eberhard — Karls — Universitat Tubingen.

Kaczanowski A., Radzikowski S., Malejczyk J. and Polakowski I. 1980: Study of intraclonal conjugation in *Chilodonella steini*. Evidence of abortive conjugation. J. Exp. Zool., 213, 261-269.

Matuszewski B. 1982: Animal cytogenetics 3 Insecta 3 Diptera I. *Cecidomyiidae*. Geb. Borntreager, Berlin-Stuttgart.

Mikami K. 1979: Intranuclear control of DNA synthesis in exconjugant cells of *Paramecium caudatum*. Chromosoma, 73, 131-142.

Mikami K. 1985: Nuclear differentiation in exconjugants of *Paramecium caudatum*. Role of nuclear division in differentiation. Dev. Growth and Differ., 27, 21-27.

Mikami K. 1987: Macronuclear development and gene expression in the exconjugants of *Paramecium caudatum*. Dev. Biol., 123, 161-168.

Mikami K. and Ng S. F. 1983: Nuclear differentiation in *Paramecium tetraurelia*. Transplantation of vegetative micronuclei into early exconjugants. Exp. Cell Res., 144, 25-30.

Nanney D. L. 1953: Nucleo-cytoplasmic interaction during conjugation in *Tetrahymena*. Biol. Bull., 105, 133-148.

Nanney D. L. 1980: Experimental ciliatology. An introduction to genetic and developmental analysis in *Ciliates*. Wiley New York.

Radzikowski S. 1972: DNA synthesis level and the occurrence of a third pregamic division as controlled by the position of nucleus in the cell. Bull. Acad. Pol. Sci., 20, 493-496.

Radzikowski S. 1985: Replication, division and mechanisms controlling the variable DNA content in the heteromeric macronucleus of *Chilodonella steini*. Arch. Protistenk., 130, 381-396.

Radzikowski S. and Gołembiewska M. 1977: *Chilodonella steini*. Morphology and culture method. Protistologica, 3, 381-389.

Raikov I. B. 1982: The protozoan nucleus. Morphology and evolution. Wien-New York, Springer.

Simon E. M. and Doerder F. P. 1981: The unique position of the degenerating macronucleus in *Tetrahymena tropicalis*. J. Protozool., 28, 203-205.

Sołtyńska M. S. 1971: Morphology and fine structure of *Chilodonella cucullulus* cortex and cytopharyngeal apparatus. Acta Protozool., 9, 49-82.

Tobler H., Smith K. P. and Ursprung H. 1973: Molecular aspects of chromatin elimination in *Ascaris lumbricoides*. Dev. Biol., 27, 190-203.

Weiske-Benner A. and Eckert W. A. 1987: Differentiation of nuclear structure during the sexual cycle in *Tetrahymena thermophila*. II. Degeneration and autolysis of macro- and micronuclei. Differentiation, 34, 1-12.

Yanagi A. and Hiwatashi K. 1985: Intracellular positional control of survival or degeneration of nuclei during conjugation in *Paramecium caudatum*. J. Cell Sci., 79, 237-246.

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Genetic and Epigenetic Factors Controlling Protrusion Occurrence in cda A1 Mutant of *Tetrahymena thermophila*

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Synopsis. The cda A1 (cell division arrest) mutant of *T. thermophila* at restrictive temperature displays an irreversible arrest of cytokinesis while other processes of divisional morphogenesis continue. In result of repetition of cell-cycles in cda A1 cells kept at 35°C, all cells eventually transform into the polynuclear monsters. The global arrest of cytokinesis in cda A1 cells grown at 35°C is accompanied with mutual relocations of cortical parts and with active, local growth of the cell cortex resulting in an appearance of the tridimensional cell surface protrusions.

Data presented in this paper show that protrusion occurrence, although causally related to the expression of the cda A1 mutated allele, may be experimentally enhanced, or diminished by internal or external factors even in monsters. Protrusions that are formed exclusively in cda A1 cells exponentially growing (but not in stationary phase), appeared in nearly all cells that were subjected to a drastic shift up from low permissive (10°C) to restrictive (35°C) temperature.

Occurrence of protrusions is, however, diminished in cda A1 exponentially growing cells if the shift up to temperature was mild (30-35°C), or in presence of the second, unlinked to cda A1 conical (co) mutation.

These studies revealed that expression of the cda A1 mutation always results in monster formation, but in defined conditions may also demonstrate another specific effect — that is appearance of the tridimensional surface corrugations.

It has been established in many experimental cell models that prior to cytokinesis, the cell surface and cortical properties of the cytoskeleton change in a predictable way and these changes are temporally con-

certed with each other (Conrad and Rappaport 1981, Schröeder, 1981).

The genic control of cytokinesis has been established in a ciliate *Tetrahymena thermophila*. Frankel et al. (1976b) produced several conditional, thermosensitive mutants of *Tetrahymena thermophila*, inhibited in cytokinesis.

In one of these mutants — the cda A1 (cell division arrest), at restrictive temperature cytokinesis is permanently blocked while mitotic cycles may still continue (Frankel et al., 1976 a, b, 1977). Full expression of this mutation at 40°C brings about the global inhibition of fission furrow while some cortical growth continues as evidenced by the appearance and outgrowth of cortical protrusions (Frankel et al., 1977; Bużańska et al., 1989). Partial appearance of a fission furrow in the form of the ventro-dorsal gradient of its expression has been observed either at lower temperature of 35°C in cda A1 (Kaczanowska et al. in prep.) or at 40°C in another allele of this locus (cda A2) (Ng and Frankel 1977).

The aim of the present paper is to find out conditions that promote or inhibit protrusion occurrence at restrictive temperature in cda A1 cells and to test the putative associations between cortical morphogenesis and occurrence of protrusions. For this purpose two factors have been tested, namely the regime of food supply and the thermic regime. It is interesting to know whether the non-cycling, starving cda A1 cells that are not able to divide, but still are able to perform another kind of morphogenesis, namely oral replacement (OR) (Frankel and Williams, 1973), are forming protrusions at restrictive temperature.

Morphological shape deformation, including appearance of short tridimensional cell surface corrugations has been induced even in a wild type *Tetrahymena pyriformis* after applying a sudden shift up of temperature in some experimental conditions (Rosenbaum et al., 1966; Erwin, 1970). The other possibility is that preincubation in a low temperature might affect stability of microtubular cytoskeleton and then affect pattern of cortical growth in cda A1 cells brought into restrictive temperature. Therefore the effects of pure temperature shift up at permissive range of temperatures on expression of protrusions in cda A1 cells were separately tested and compared with the respective sudden and mild shifts up to the restrictive temperature.

The unlinked to cda A1 gene, namely conical (co), in homozygosity in *T. thermophila* brings about the nonconditional, permanent change of the cell shape. It does not affect the course of divisional morphogenesis and OR, but it causes an unequal partitioning of cell during cytokinesis into large anterior and small posterior daughters (Doerder

et al., 1975; Schaffer and Cleffmann, 1982). It rises a question whether expresssion of these gene may affect or modify expression of protrusions brought by homozygosity of cda A1 allele.

Material and Methods

Tetrahymena thermophila strains used in this study were WU 60 (single homozygous for cda A1 strain) (Gaertig et al., 1988), B 367 (double homozygous for cda A1 and co) (Doerder et al., 1975) and Cu 399 (cy-sens) as a wild type control in respect to cda and co alleles. Culture cells were kept at room teperature (22°C). Growing medium contained 1% PP, 0,5% yeast extract (PPY) and Fe^{3+} at 9×10^{-5}M (Orias and Bruns, 1976).

In all experiments 24 h schedule was chosen. At O time rutinely logarythmically growing cells (density 5×10^4 cells/ml) or stationary cells (starved overnight in pure Tris HCl pH-7,4), were splitted into two experimental flasks and incubated at 30°C and 10°C (permissive temperatures) for 8 h. After this time all cells were transferred to 35°C (restrictive temperature) for 16 h.

In another experimental model WV 60 and Cu 399 logarythmically growing cells after 8 h in 10°C (low permissive temperature) were shifted to 30°C (high permissive temperature) for 16 h. That experiment served as a control for a possibility of nonspecific shape change unrelated to the expression of the cda A1 mutation.

Quantitative light microscopic (Carl Zeiss Jena) estimations were made on a formaline fixed samples. Protargol slides according to Ng and Nelsen (1977), with modification (Bużańska et al. 1989), were prepared for each experimental variant. They were used as a qualitative documentation and control adequacy of estimations made on formaline samples.

Frequency of stationary cells WV 60, B 367 and Cu 399 which undergo oral replacement in conditions of 10°C, 30°C and after temperature shifts 10°C to 35°C and 30°C to 35°C was estimated on protargol slides (n = 300 for each value).

Values shown in Table 3 expressed as a percent of cells with projections are averaged from three independent experiments. In each experiment 300 cells were counted, thus for each value shown the total number of specimens examined was 900.

Classification of morphological groups used in this study was based on formalin and protargol observations. A category of "small protrusions" accounted for those protrusions which were no longer than 1/4 of the body cell length. In other cases protrusions were categorized as "long protrusions". For statistical evaluation of data t-Student test was used (Sokal and Rohlf 1969).

Results

Comparison of the detectability of protrusions in the two samples fixed with either in formalin or fixed with osmium and stained with protargol

In the formalin fixed and protargol stained samples, deriving from the same experiment, the number of cells which are forming protrusions (as defined in Materials and Methods) is compared (Table 1).

Table 1

Mean percentage of exponentially growing cda A1 cells, forming protrusions at restrictive temperature, revealed by formalin staining and protargol technique ($n = 300$)

	Temperature shift	
	10–35°C	30–35°C
Formalin fixed sample	96,3%	42,4%
Protargol stained sample	98%	45%

Light microscope observations and quantitative estimations of cells with projections in samples fixed with formalin and stained with protargol proved to be not statistically different ($p \leqslant 0.01$). This proves that both methods are equivalent for detection of cells with protrusions.

Oral replacement in stationary cda A1 and control cultures in different temperature conditions.

Cells kept at stationary phase are not able to divide, however, they can still perform repeatedly some morphogenetic activity in a form of the oral replacement process (OR) (Frankel and Williams 1973). To test whether this activity may be related to protrusion formation, the percentage of cells being in OR has been evaluated.

Results presented in Table 2 show no statistic difference of occurrence of OR in different strains. The percentage of cells in OR does not changes after shifting of cells either from 10°C or 30°C to restrictive

Table 2

Frequency of stationary cells performing the oral replacement (OR) at different temperature conditions ($n = 300$)

	Mean % of cells in OR incubated 8 h in		Mean % of cells in OR kept 16 h at 35°C	
	10°C	30°C	10–35°C	30–35°C
cda A1	2%	1.5%	1%	1%
co, cda A1	3%	2.6%	1%	2%
Cu 399	1.5%	1.1%	1.5%	1.5%

temperature. Therefore any appearance of protrusions in stationary cells at restrictive temperature in fraction of cells statistically higher from 1-3% may prove that this process is not necessarily related to the actual OR.

Temperature and nutrient conditions for appearance of tridimensional surface deformations in cda A1 mutant of *Tetrahymena thermophila*

Some specificity of the phenotypes of cells carrying cda A1 allele has been dissected in this study for certain experimental conditions and may be summarized as follows:

Cda A1 *Tetrahymena thermophila* cells grown at permissive temperature (Pl. I 1) look like wild-type cells. Morphostatic cell is of ovoidal shape with the oral apparatus located on the anterior ventral part of the cell, two CVPs (contractile vacuole pores) located at the lateral posterior part of the cell and the ciliary rows (composed of the ciliated and not ciliated basal bodies) running along the cell body. Dividing cda A1 cell in permissive temperature double their cortical organelles (oral apparatus, CVPs and b. bodies) in a segmental manner (Tartar, 1962; Frankel et al. 1981).

Cda A1 cells at restrictive temperature continue their cortical growth and nuclear divisions but do not form fission furrows (Frankel et al., 1976a, 1977). Then eventually the monster cells of a diverse morphology are formed. Four different phenotypes are dissected in this study: (1) wild type; (2) altered shape, with more than 2 OAs per cell but with no protrusions. They are designated as monsters without protrusions; (3) altered shape, increased number of OAs and only small protrusions (length of protrusions does not excess the length of 1/4 cell). They are designated as "monsters with small protrusions"; (4) altered shape, increased number of OAs per cell, forming long protrusions. Designated as "monsters with long protrusions".

Frequency of appearance of different phenotypes is presented in Table 3.

Indicated strains either fed or starved (in logarythmic or stationary phases) were subjected to different temperature regimes. Experimental cells were shifted from either 10°C or 30°C (low and high permissive temperature) into 35°C (restrictive temperature) for 16 h. By that time all cda A1 cells, either those which were subjected to 35°C after their temperature sensitive period in the cell cycle (Frankel et al., 1980 a, b) and finished their division at 35°C, or those which were inhibited in furrowing immediately, formed monsters (cells with altered shape and kinetosomal displacements on the surface).

Table 3

Frequency distributions of different phenotypes in three *T. thermophila* strains subjected to different temperature and feeding conditions

	Temperature regime		Phenotypes			
			wild type (control)	monsters without protrusions	monsters with small protrusions	monsters with long protrusions
Cu 399 (wild type) logarythmic	controls	10°C–30°C	100%	–	–	–
		10°C–35°C	100%	–	–	–
cda A1 logarythmic		10°C–3°C	100%	–	–	–
cda A1 stationary		10°C–35°C	98%	2%	–	–
		30°C–35°C	96%	4%	–	–
co, cda A1 stationary		10°C–35°C	96%	4%	–	–
		30°C–35°C	97%	3%	–	–
cda A1 logarythmic		10°C–35°C	–	1.4%	–	98.6%
		30°C–35°C	–	55.3%	44.7%	–
co, cda A1 logarythmic		10°C–35°C	–	26.8%	73.2%	–
		30°C–35°C	–	95%	5%	–

Blocked data are statisticaly different on level $p \leqslant 00.1$

Normal (control) morphology (Pl. I 1) was obtained in 100% in test with wild type Cu 399 cells shifted either from 10°C to 30°C or from 10°C to 35°C and for exponentially growing mutants transferred from low permissive (10°C) to high permissive temperature (30°C). The last experiment was designed to test the possibility of non-specific shape changes and protrusion formation in wild type.

Next experiment concerned transfer of mutated strain from low to high permissive temperature and was aimed to test the effect of drastic shift up of temperature on the expression of cda A1 phenotype. Results of three experiments yielded only normal phenotypes. Thus shift of temperature does not affect morphology of wild type strain or cda A1 strain if not subjected to restrictive temperature.

Predominantly wild type phenotype (96-98%) was also obtained in cells being at stationary phase (kept 24 h in Tris buffer) and then transferred from either 10°C or 30°C to restrictive temperature. Apparently in these not-cycling cells the normal morphology of cells is kept. Thus cda A1 and co, cda A1 cells starved in Tris buffer do not form protrusions even at restrictive conditions.

Combination of optimal nutrient conditions and application of restrictive temperature always promote two morphological effects in cells

carrying cda A1 allele: these cells are able to form monsters and they are able to form and maintain cortical protrusions. Nevertheless the frequency of appearance of monsters with protrusions drastically depends upon the temperature regime. Mutant cells, which are cycling at restrictive temperature but do not form protrusions, are represented mostly (55,3%) by cda A1 cells transferred from 30°C to 35°C (mild shift of temperature). The frequency of this phenotype for the same temperature shift is enhanced in cells carrying also conical (co) allele (95% of monsters with no protrusions — Pl. I 2).

Remaining fractions of cells in both experiments revealed phenotype of monsters with small protrusions (44.7% for cda A1 cells and 5% for co, cda A1 cells — Pl. I 3).

However, it happened that drastic temperature shift from 10°C to 35°C invitably produces monsters with long protrusions in cda A1 logarythmically growing cells (98.6% — Pl. I.4). Again the presence of co allele dampened the effect of drastic shift up of temperature (for the transfer from 10°C to 35°C only monsters with small protrusions were obtained).

In all experiments there is a statistically different distribution of fractions of different phenotype depending upon the drastic (10°C-35°C) or mild (30°C-35°C) shift of temperature, even though the restrictive temperature was the same.

Discussion

In a conditional thermosensitive mutant cda A1 (cell division arrest) of *Tetrahymena thermophila*, the mutational arrest of cytokinesis at restrictive temperature (35°C and higher) is subsequently followed by the transformation of cells into monsters. Many of these cells displaying various monstrous shapes and patterns were often provided with the funger-like protrusions of cortex, preferentially localized in the areas of mutationally inhibited fission-line zones (Frankel et al., 1977). It has been shown that these protrusions represent the local, autonomous centres of the cell cortex growth (Bużańska et al., 1989).

Previously Rosenbaum (1966) and Erwin (1970) have reported the temperature-induced deformations of the cells of another *Tetrahymena* species, namely *Tetrahymena pyriformis*. Structural changes included formation of corrugations over the cell surface, multiple invaginations of the alveolar membranes and local displacements of the basal bodies (Lo et al., 1976). These deformations, although spectacular, did not involve subsequent activation of the local cortical growth and these

effects were observed to occur only at sublethal temperature (40°C) and in medium supplied with some phospholipids.

In the present studies, performed on *Tetrahymena thermophila* grown at low or optimal temperatures (below 40°C) such effects were not detected. These effects were not observed also in mutated cda A1 cells shifted from low (10°C) to optimal (30°C) temperatures. Thus both in a wild type and mutated strains of *Tetrahymena thermophila*, temperatures below threshold for expression of mutational deffect did not induce non-specific shape alternations.

Certain conditions were nacessary for induction of protrusion outgrowth in cda A1 cells: (1) good nutrient conditions (cells should be cycling), (2) preincubation of cells in a low permissive temperature before transferring into restrictive temperature.

The effect of protrusion formation was dumped (or even inhibited in a stationary cells) by the use of another, unlinked to cda A1 mutation, namely conical (co), or by the application of a mild temperature shift (from 30 to 35°C).

Thus the method dissecting two morphological aspects of expression of cda A1 mutation, namely smooth monster formation and monster formation with occurrence and maintenance of protrusions was devised.

Results of these studies clearly show that the process of protrusion occurrence, which appears only in cycling cda A1 cells is sensitive to the regime and shift up of temperature. It raises a question about the role of this shift up temperature regime on expression of the cda A1 mutation.

The role of preincubation at low temperature for expression of protrusions may depend on structural destabilization of membrane and cytoskeleton in cells adopted to low temperature and then brought up to high temperature. First the essential role of thermic shift up applied to *T. thermophila* has been shown to affect cell membrane chemistry in terms of phospholipid composition and its fluidity (N o z a w a and T h o m p s o n, 1971; N o z a w a and K a s a i, 1978). The other possibility may concern the stability of microtubular cytoskeleton. At low temperature some microtubules tend reversibly to depolimerize (S a m a k et al., 1987). This instability may affect morphogenesis at high temperature in cda A1.

The evidence of the effect of the expression of another unlinked mutation conical on cda A1 induced course of monster formation allows to dissect processes related exclusively to inhibition of cytokinesis from the release of the active autonomous growth of parts of *Tetrahymena* cortex based on microtubules.

To our knowledge, an induction of dispersed centres of the cytoskeletal growth has been detected only in cda A1 mutant of *Tetrahymena*

thermophila. Therefore it is not excluded that this effect is directly and intimately related to the cda A1 gene product defect.

At restrictive temperature the cda A1 mutation brings about irreversible arrest of cytokinesis. The monster formation is the subsequent developmental effect related to the relocations of some parts of cortex against the others. Autonomy of some parts of cortex and relocations of others supplemented with irregular local active cortical growth in a form of protrusions, altogether leads to monstrosity of polynuclear cda A1 specimens. In this paper an occurrence and maintenance of protrusions in monsters may be experimentally dissected from other aspects of monstrosity. It was shown, that an active growth of the parts of the cortex can be controled by genetic or epigenetic factors.

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REFERENCES

Bużańska L., Gregory D. W. and Wheatley D. N. 1989: Protrusion formation in the cell division-arrested mutant *Tetrahymena thermophila* cda A1, and the elucidation of some rules governing cytoskeletal growth. J. Exp. Zool., 251, 27-36.

Conrad G. W. and Rappaport R. 1981: Mechanisms of cytokinesis in animal cell. In: Mitosis/Cytokinesis (Ed. A. M. Zimmerman and A. Forer), Acad. Press 365-391.

Doerder F. P., Frankel J., Jenkins L. M. and De Bault L. E. 1975: Form and pattern in ciliated protoza: analysis of a genic mutant with altered cell shape in *Tetrahymena pyriformis*, Syngen 1. J. Exp. Zool., 192, 237-258.

Erwin J. A. 1970: The reversal of temperature-induced cellsurface deformation in *Tetrahymena* by polyansaturated fatty acids. BBA, 202, 21-34.

Frankel J., Jenkins L. M. and De Bault L. E. 1976a: Causal relations among cell cycle processes in *Tetrahymena pyriformis:* An analysis employing temperature sensitive mutants. J. Cell Biol., 71, 242-260.

Frankel J., Jenkins L. M., Nelsen E. M. and Doerder F. P. 1976b: Mutations affecting cell division in *Tetrahymena pyriformis*. I. Selection and genetic analysis. Genetics, 83, 489-506.

Frankel J., Mohler J., and Frankel A. K. 1980a: Temperature sensitive periods of mutations affecting cell division in *Tetrahymena thermophila*. J. Cell Sci., 43, 59-74.

Frankel J., Mohler J., and Frankel A. K. 1980b: The relationship between the excess-delay phenomenon and temperaturesensitive periods in *Tetrahymena thermophila*. J. Cell Sci., 43, 75-91.

Frankel J., Nelsen E. M. and Jenkins L. M. 1977: Mutations affecting cell division in *Tetrahymena pyriformis*, syngen 1. II. Phenotypes of single and double homozygotes. Develop. Biol., 58, 255-275.

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The Synthesis and Turnover of the Cellular Matter of Ciliates in the Rumen

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Synopsis. The synthesis, outflow and degradation of the protozoal matter across the forestomachs was examined in the sheep fed hay-concentrate diet every 12 h. Pool size of the synthesised matter was calculated from dry weight of the ciliate cells, their number and growth rate. Pool size of protozoal matter in the rumen and also of that leaving the forestomachs was calculated from dry weight and number of ciliates.

Dry weight of single ciliate varied from about 5 to over 400 ng in relation to the cell dimension, and doubling time of protozoa was 7.2-10.3 h. Protozoal matter pool in the rumen varied between 39 and 54 g dry matter (DM) and was not related to the total protozoa counts. The ciliates constituted 18-32% of the total microbial biomass in the rumen of sheep. The rate of gross synthesis was 38-52 g DM/12 h in relation to ruminal pool size of protozoa. The outflow rate was about 11-14 g and degradation rate 25-39 g DM/12 h. A negative correlation was found between efficiency of organic matter (OM) digestion and pool size of protozoa leaving the forestomachs. The correlation between turnover rate of rumen fluid and both the gross synthesis and a flow of protozoal matter to abomasum was positive. Pool size of ciliates degraded in the forestomachs was positively correlated with efficiency of OM digestion and negatively with turnover rate of rumen fluid. Synthesis, outflow and degradation of protozoal matter with respect to different groups of ciliates was also measured.

Pool size of N utilized by ciliates for their growth was 3.5-4.8 g i.e. 26-36% of the dietary N. Pool size of protozoal N entering the abomasum was only 1.0-1.2 g/12 h and constituted about 13-21% of microbial N leaving the forestomachs. Pool size of the protozoal N recycled in the forestomachs was 2.4-3.6 g/12 h and it was equivalent to 18-27% of the dietary N. Protozoal amino acids contributed to 12-22% of microbial amino acids in the omasal effluent.

The efficiency of protozoal protein formation varied in a range of 2.1-4.5 g N/kg OM apparently digested in the forestomachs or in range

of 10.5-13 g N/kg OM digested by ciliates themselves. Appropriate results related to bacteria were 12.8-17.6 and 19-37 g N/kg OM apparently digested in the forestomachs and by bacteria themselves respectively.

It was calculated that ciliates contributed to 31-36% of OM digested across the forestomachs sheep.

"Rumen protozoa present perhaps the greatest challenge to rumen microbiologists" (Hungate 1986). This opinion seems to be still actual however, methodical difficulties limit the attractivenes of these organisms for study especially as far as experiments performed in vivo are concerned. In fact some progress has been recently noted in relation to synthesis of ciliate matter (protein) in the rumen (Leng et al. 1981, 1984, 1986, Leng 1982 Ffoulkes and Leng 1988). However, comparative studies carried out with an use of more direct methods are still of value.

The main difficulty of the research is the quantification of the outflow of protozoal matter from the rumen because of a failure of any direct methods. It was calculated as a difference between total microbial and bacterial flow (Harrison et al. 1979, Steinhour et al. 1982; Meyer et al. 1986) or deducted from quantity of aminoethylphosphonic acid (AEPA) in duodenal digesta (Whitelow et al. 1984). A rate of the flow of protozoal protein at duodenum of sheep was found there as 2-5 g N/day. On the contrary Weller and Pilgrim (1974) estimated it as 0.14-0.44 g N/day only. This last flow was calculated more directly i.e. from the number of ciliates passing down at the omasal canal.

It is well documented that protozoa are very active group of rumen microorganisms and are able to utilize most of the nutrients of the ruminants food (Coleman 1983, 1984, Forsberg et al. 1984, Williams et al. 1984) but their contribution to the bulk of the organic matter converted in the rumen ecosystem was deducted from in vitro experiments (Demeyer 1981, Akin 1986) or from comparing the faunated and ciliate-free ruminants (Kayouli et al. 1984). It is also well known that protozoa are selectively retained in the rumen (Weller and Pilgrim 1974, Michałowski et al. 1986). It presumably affects the efficiency of net synthesis of protozoal protein which was, however, only poorly quantified (John and Ulyatt 1984) and therefore further studies are still nesessary.

The present paper reports the results of studies performed for getting more direct informations about a contribution of ciliates to the matter turnover in rumen ecosystem in relation to their biomass and witn comparison to bacteria. Therefore following estimations were undertaken: (i) pool size of protozal and bacterial matter in the rumen; (ii)

a rate of protozoal matter synthesis and degradation across the forestomachs; (iii) protozoal and bacterial matter flow at the abomasum and efficiency of protozoal and bacterial protein net synthesis; (iv) a contribution of ciliates to utilization of dietary N and dietary organic matter; (v) importance of rumen protozoa as an amino acid source for the host. The basis of all estimations was a number of ciliates and their chemical composition. The results concerning the concentration of protozoa in the rumen and in the digesta leaving the forestomachs of sheep used for calculations in this report were published earlier (Michałowski et al. 1986).

Material and Methods

Six growing sheep, 9-11 months old, 30-40 kg live weight and two adult sheep weighing 70 and 75 kg were used in experiments presented in the paper. The animals were kept in separate pens and given pelleted concentrate (29.6 g N/kg DM) and hay (15.2 g N/kg DM). Organic matter constituted 79.5 and 83.4% of the dry matter (DM) of the mentioned food components. The sheep were fed every 12 h. Growing animals received 350 g concentrate and 300 g hay per meal while the adult — 700 g concentrate and 600 g hay, respectively. Water was available all the time. The animals were given the diet at least 4 weeks before the experiments started.

Samples of rumen content (200-300 g) and omasal effluent (not less than 50 g) were collected from three growing sheep (Nos. 2, 4 and 5) equipped with rumen and abomasal canula. Omasal effluent was collected via a nylon-texture rubber sleave saturated to the omasum-abomasum orifice (Michałowski et al. 1986). The subsamples for protozoa counts (5 g) and for chemical analysis (10-25 g) were then weighed out in duplicate from the collected material while the remaining part was turned back into the rumen. The collection was performed just before morning feeding and at 2, 5, 8 and 12 h thereafter. It was repeated 3-6 times on 3-6 different days. The collection started 2 weeks after beginning the infusion of CrEDTA (21.4-24.0 mg Cr/h) into the rumen of each animal. CrEDTA was infused in order to determining the rumen fluid and omasal effluent volume. An infusion of a hypertonic salt solution was also performed periodically in order to increase rumen fluid turnover rate. Detailed description of this part of experiments was presented earlier (Michałowski et al. 1986).

Growth rate in ciliate number was measured by a "zero-time-rate" method in vivo and in vitro. Three growing sheep (Nos. 6, 7 and 8) equipped with rumen canulae (2.8 cm internal diameter) were used for in vitro measuring. Approximately 200 g content was taken from the rumen. Two equal samples (25 g) were then weighed out, introduced into the glass centrifuge tubes and incubated under anaerobic conditions for 1 h in water bath at 38°C. Samples for protozoa counts (5 g) were taken before the comencement and at the end of each incubation period. Anaerobic conditions inside the glass tubes and a mixing of their content ensured a CO_2 stream bubbling from the bottom of the tubes at the flow intensity of 30-50 cm^3/min. The samples for incubation were taken from the rumen every 2 h over 12 h period and the first sample was taken before morning feeding. Experiments were repeated two times.

Growth rate of ciliates in the rumen was measured using two adult sheep equipped with large (10-12 cm internal diameter) rumen fistulae and "nylon bags" were used for incubation of the samples of rumen content. Preparation and weight of incubated samples were the same as described above. However, a suspension (1 ml) of DOWEX balls of 30-50 μm diameter was added before the rumen content was introduced into the bags and the first sample for protozoa counts was taken. The bags were put into ventral sac of the rumen by hand and incubated there for 2 h. A weight of 100-130 g was connected to the bags in order to keep them in ventral sac. Samples (5 g) for protozoa and DOWEX balls counts were taken just before and at the end of each incubation period. DOWEX balls were suspended in 36000 g rumen fluid supernatant fraction and added as a marker for estimation of changes in volume of the samples during incubation in the rumen. The bags used for incubation were made from a nylon texture (Reichelt, RFG) of about of 5 μm of pore size according to Jouany and Senaud (1979). Experiments were repeated five times.

Protozoa free from bacteria and from food debris were prepared by sedimentation method. Portions of rumen fluid (300-400 ml) were taken from the rumen of growing sheep before feeding, strained through 4 layers of surgical gause and incubated in a glass beaker for 30 min at 38°C. Supernatant was then withdrawn by suction and discharged while deposit, containing protozoa was resuspended in a warm (38°C) "caudatum type" salt solution (Coleman et al. 1972) and incubated again. The washing procedure was repeated 4-5 times until the sediment consisted only of protozoa. Clean ciliates were resuspended in 25 ml of formaldehyde solution (1% w/v) and separated to the groups of different cell dimensions according to their gravity. Preliminary separated groups were then given to further cleaning while other ciliates were separated out using different sedimentation rate. Finally the protozoa were washed two times with a cold distilled water and resuspended in its small volume (15 ml). The volume of suspension was precisely measured and the ciliates were dried to constant weight after sampling for protozoa counts.

Bacteria were obtained from supernatant fraction of rumen fluid after removal of ciliates and food debris by centrifugation the samples at about 900 g for 10 min. Supernatant fraction was collected by suction and centrifuged again at 36 000 g for 30 min. The sediment was then washed twice with a distilled water and dried to constant weight.

All samples of protozoa were fixed with 4% (w/v) formaldehyde solution. The ciliates were counted in Fuchs-Rosenthal counting chamber or by the method described previously (Michałowski 1975) in relation to density of ciliate group. Each sample was six times counted, however, the samples of clean ciliates and the protozoa from "growth experiments" were estimated not less than ten times and not less than 2000 cells per sample per dimension group was counted. Identification of ciliates was performed according to Dogiel (1927) and Grain (1966).

Amino acids and 2,6-diaminopimelic acid (DAPA) were estimated separately after hydrolysing the dry samples with 6N HCl at 110°C for 16 h and at 105°C for 20 h respectively. The hydrolysates were evaporated under reduced pressure, washed two times with distilled water and dissolved in 0.2 M citrate buffer (pH 2.1), then filtered through a paper filter and analysed with an use of Durum automatic amino acid analyser.

Total N was measured by micro-Kjeldahl method. Cr was estimated by atomic absorption spectrometry (Perkin Elmer 400) using supernatant fraction of rumen content and omasal effluent (Michałowski et al. 1986).

Dry matter of rumen content, omasal effluent and rumen microorganisms was obtained by freeze-drying for 36 h and organic matter (OM) by ashing of dry samples in muffle furnace at 550°C for 12 h.

Number of ciliates in whole rumen and these leaving the forestomachs was the basis of calculation of the pool size of protozoal matter. Both of them were calculated from concentration of ciliates and volume of appropriate digesta. Rumen content volume before feeding and abomasal flow were calculated from volume of liquid fraction and its proportion in the digesta. Rumen liquid fraction was computed from Cr concentration versus infusion rate and time of infusion. A detailed description of all calculation is given in the report concerning the concentration of ciliates in the rumen content and omasal effluent of the same sheep (Michałowski et al. 1986). Rumen content volume after feeding was calculated from pool size of dry matter (DM) and its proportion in the digesta (see Discussion). Dry matter pool size of rumen content after feeding was calculated from the equation:

$DM_t = (DM_o + DM_f) - (DM_e + DM_d)$ where DM_t is dry matter pool size in the rumen at the "t" time after feeding; DM_o is pool size of dry matter of rumen content before feeding; DM_f is dry matter of the food ration; DM_e is dry matter of the omasal effluent leaving the forestomachs at "t" time; DM_d is the pool size of dry matter digested in the forestomachs at "t" time. Pool size of DM digested in the rumen was calculated on the basis of an assumption that $DM_d = DM_f - DM_e$, according to Egan (1974).

Pool size of DM digested in the rumen at "t" time was calculated from equation $DM_d = DM_{d12} \times fD$; where DM_{d12} is a pool size of dry matter digested in the rumen over whole feeding cycle (g/12 h) and fD is a digestion factor calculated from production rate of volatile fatty acids (VFA) in the rumen of the same animals (Michałowski 1987). It was calculated on the basis of assumption that ratio of VFA produced/DM digested in the rumen (mol/kg) is constant (Czerkawski 1986).

DM_e was calculated from total flow of DM at abomasum (Michałowski et al. 1986), infusion rate of CrEDTA into the rumen and concentration of the marker in omasal effluent at different time after feeding.

Digestion of organic matter was calculated from pool size of OM in the ration and in omasal effluent similar to digestion of DM (see above).

The apparent digestibility of OM across the forestomachs was calculated according to Egan (1974).

All statistical analysis were made according to Ruszczyc (1970).

Results

Rumen microfauna of sheep consisted of ciliates of following species: *Entodinium exiguum, Entodinium minimum, Entodinium nanellum, Entodinium simplex, Entodinium caudatum, Entodinium longinucleatum, Entodinium bursa, Anoplodinium denticulatum, Diploplastron affine, Polyplastron multivesiculatum, Ostracodinium triloricatum, Ophryoscolex cau-*

datus, Dasytricha ruminantium, Isotricha prostoma and *Isotricha intestinalis.*

Separation of ciliates according to their gravity resulted in three groups of these organisms (Table 1). The group "small" ciliates consisted of entodinia (except *Entodinium bursa*) and *Dasytricha ruminantium.* The

Table 1

Dry weight of single ciliate from three dimension groups (ng), and contribution of N (%) to protozoal and bacterial dry matter. (Mean values ± Standard error, S. E.)

Microorganisms	Dry matter DM		N	
	Mean	S.E.	Mean	S.E.
"Small" ciliates	4.8	0.31	9.6	0.51
"Medium" ciliates	47.2	4.81	9.4	0.39
"Large" ciliates	401.8	22.83	8.6	0,31
Bacteria	not estimated		8.2	0.18

group "medium" ciliates consisted of *Entodinium bursa* occurring in very small number, and diplodinia excluding *Polyplastron multivesiculatum.* This last species together with *Ophryoscolex caudatus* and both species of *Isotricha* constituted the group of "large" ciliates. Dry weight of single ciliate from this last group was over 80 times greater than dry weight of "small" protozoon and over 8 times than "medium" ciliate ($p < 0.001$) but the N proportions in protozoal matter were similar. A contribution of nitrogen to bacterial dry matter is also given in Table 1.

Significant differences between amino acid composition of ciliates from particular groups were not found. Thus only the mean values are given together with results concerning bacteria as well as findings of some other authors (Table 2).

A rate of increase in ciliate concentration (Table 3) varied in range of 6.8-9.9%/h ($P > 0.05$). The presented data are mean values of 12 h feeding cycle because significant daily variations were not found. The doubling time of ciliates (Table 3) was calculated from the equation $N_t = N_o (1 + k)^t$ where N_t is concentration of ciliates at "t" time after feeding; N_o is number of ciliates before feeding; k is a growth rate factor, i.e. 0.068, 0.075, 0.088 and 0.099/h and t is time after feeding (h). In spite to an increase in ciliate number observed inside the incubation tubes or "nylon bags" the concentration of ciliates in the rumen content at the end of "growth experiment days" was similar or lower than at the beginning (Table 3).

Table 2

Amino acid composition (g amino acid/100 g total amino acids) of hydrolisates of rumen ciliates and bacteria with comparison to literature data (Mean values ±S.E.)

Amino acids	Ciliates		Bacteria	
	Measured	Published[b]	Measured	Published[b]
Asparatic acid	14.4[a]±0.23	12.5±0.12	12.7[a]±0.37	11.4±0.17
Threonine	5.7 ±0.12	5.4±0.22	6.2 ±0.08	5.2±0.17
Serine	5.1 ±0.12	4.9±0.76	6.5 ±0.63	4.4±0.32
Glutamic acid	15.2[a]±0.29	14.3±0.95	11.9[a]±0.57	13.0±0.67
Proline	3.9 ±0.21	3.2±0.55	4.0 ±0.10	4.1±0.68
Glycine	4.3 ±0.21	4.5±0.24	5.4 ±0.04	5.6±0.29
Alanine	4.5[a]±0.13	4.6±0.32	7.4[a]±0.06	6.4±0.41
Valine	5.0 ±0.14	4.6±0.35	5.7 ±0.32	5.2±0.72
Methionine	1.6 ±0.19	2.0±0.31	1.3 ±0.15	2.6±0.20
Isoleucine	7.2[a]±0.17	6.1±0.43	5.8[a]±0.13	4.9±0.81
Leucine	9.2 ±0.41	8.0±0.13	8.7 ±0.18	6.4±0.87
Tyrosine	4.0 ±0.22	4.8±0.38	4.2 ±0.39	4.3±0.07
Phenyloalanine	6.0 ±0.14	5.9±0.30	5.4 ±0.34	4.9±0.12
Lysine	7.3 ±0.25	11.0±1.04	8.5 ±0.10	8.7±0.78
Histidine	1.6 ±0.22	2.4±0.43	1.7 ±0.21	3.4±1.47
Arginine	5.0 ±0.23	5.8±0.92	4.7 ±0.14	5.1±0.17
Total AA[c]	54.2 ±2.29		48.9 ±2.84	

[a] – results statistically different ($p < 0.05$), [b] – mean values from results of Hoeller and Harmeyer (1964), Purser and Buechler (1966) and Cockburn and Williams (1984), [c] – expressed as g amino acids/100 g DM

Table 3

The growth rate in ciliate number (%/h), doubling time of particular ciliate group (h) and concentration of protozoa in the rumen of the growing and adult sheep at the beginning and at the end of „growth experiment days" ($\times 10^3$/g). Mean values ±S.E.

Ciliates	Growing sheep				Adult sheep			
	Growth rate	Doubling time	Ruminal concentration		Growth rate	Doubling time	Ruminal concentration	
			initial	after 12h			initial	after 12h
"small"	8.8±1.50	8.2±1.40	458±43.2	474±22.5	9.9±1.79	7.2±1.30	597±55.8	438±94.8
"medium"	8.8±1.46	8.2±1.36	41± 3.4	49± 3.7	8.8±2.24	8.2±2.09	21± 4.6	17± 3.4
"large"	6.8±1.11	10.3±1.68	7± 2.8	6± 1.3	7.5±2.10	9.3±2.49	14± 2.0	10± 2.1

"Small" ciliates predominated in the rumen of all three animals, while the "large" protozoa constituted only 0.6-1.3% of the total counts (Table 4). Similar proportions were found also in the digesta leaving the forestomachs, however, concentration of ciliates in omasal effluent was lower by 63-70% than in rumen digesta. Data concerning these results

were discussed in previus paper (Michałowski et al. 1986). Rumen content volume increased by 21-32% during first two hours after feeding and then consecutively decreased. Mean weight of rumen digesta and omasal effluent are given in Table 4.

Table 4

The characteristic of some indices of rumen content and omasal effluent of three sheep. Mean values ±S.E.

Item	Sheep 2	Sheep 4	Sheep 5
No. of experiments	6	5	3
Weight of rumen digesta (kg)	5.6± 0.13	6.7± 0.13	5.5± 0.22
Rumen fluid turnover rate (×/d)	2.3± 0.25	1.8± 0.12	2.3± 0.11
Ruminal protozoa counts ($\times 10^3$/g)	737.0±115.80	475.0±28.10	750.0±21.00
"small" ciliates (%)	90.1± 0.59	92.6± 1.08	94.5± 0.48
"medium" ciliates (%)	8.8± 0.67	6.2± 0.98	4.9± 0.39
"large" ciliates (%)	1.3± 0.50	1.2± 0.12	0.6± 0.09
Omasal effluent flow (kg/12h)	4.9± 0.33	6.0± 0.33	5.2± 0.35
Protozoa counts in effluent ($\times 10^3$/g)	223.0± 51.70	178.0±27.20	236.0±14.20
"small" ciliates (%)	91.3± 2.44	94.9± 0.75	95.1± 0.19
"medium" ciliates (%)	7.3± 2.65	3.9± 0.64	4.2± 0.17
"large" ciliates (%)	1.4± 0.83	1.2± 0.14	0.7± 0.03

Protozoal matter in the rumen (Table 5) was calculated from ciliate number and dry weight of their cells. It constituted 18-32% of the microbial matter of rumen microorganisms (bacteria and ciliates) and was relatively constant over all 12 h feeding cycle. Any correlation between total number of ciliates and pool size of protozoal matter was not faund ($p > 0.05$) similar as correlation between this second value and turnover rate of rumen fluid. Contribution, however, of "large" ciliates to protozoal matter pool decreased while this of "small" and medium" ciliates

Table 5

Pool size of protozoal and bacterial matter in the rumen of sheep, rate of gross synthesis of protozoal matter in the rumen and N pool utilized by ciliates for their growth

Item	Sheep 2	Sheep 4	Sheep 5	Standard error
Number of experiments	6	5	3	
Pool size of protozoal matter (g DM)	53.5	40.2	38.8	2.86
Pool size of bacterial matter (g DM)	114.5	180.4	97.9	10.53
Rate of gross synthesis (g protozoal DM/12h)	51.9	37.7	38.3	2.58
N pool utilized by ciliates for growth (g N/12h)	4.8	3.5	3.6	0.24

increased with an increase in dilution rate of rumen fluid (Fig. 1). A decrease in pool size of matter of "large" ciliates and an increase in this of "small" and "medium" protozoa was also observed there.

The bacterial matter in the rumen was calculated from the amount of 2,6-diaminopimelic acid (DAPA) in the rumen content and concentra-

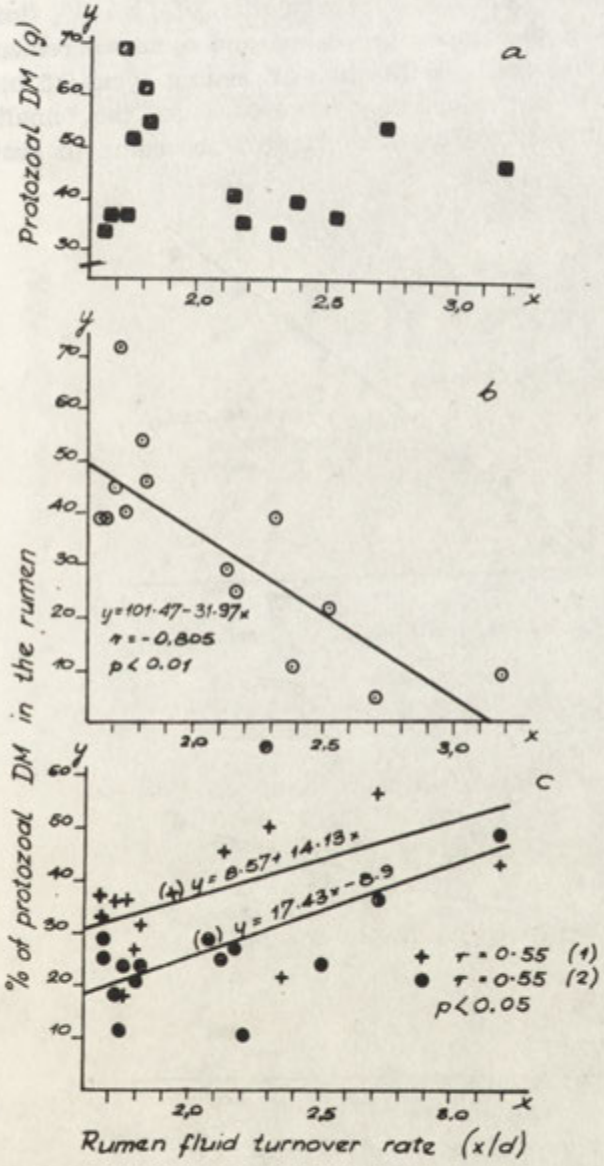

Fig. 1. Ruminal pool size of protozoal matter of sheep (a), and relationship between turnover rate of rumen fluid and contribution of "large" (b) as well as "small" (1) and "medium" (2) ciliates (c) to protozoal matter pool in the rumen

tion of this natural marker in bacterial matter. Bacteria constituted 68-82% of total microbial matter in the rumen (Table 5) and concentration of DAPA in dry bacterial matter was 4.1 (S. E. 0.19) mg/g.

Gross production (GP) of protozoal cellular matter was calculated from equation $GP = [(B_s \cdot c_s) + (B_m \cdot c_m) + (B_l \cdot c_l)] \cdot t$ where B_s, B_m and B_l are mean pool sizes of the matter of "small", "medium" and "large" ciliates in the rumen (g); c_s, c_m and c_l are increment factors in their biomass (x/h) and t is the time of feeding cycle (12 h). The increment factors used for calculations were 0.088 for the "small" and "medium" ciliates and 0.068 for these "large" according to growth rate in

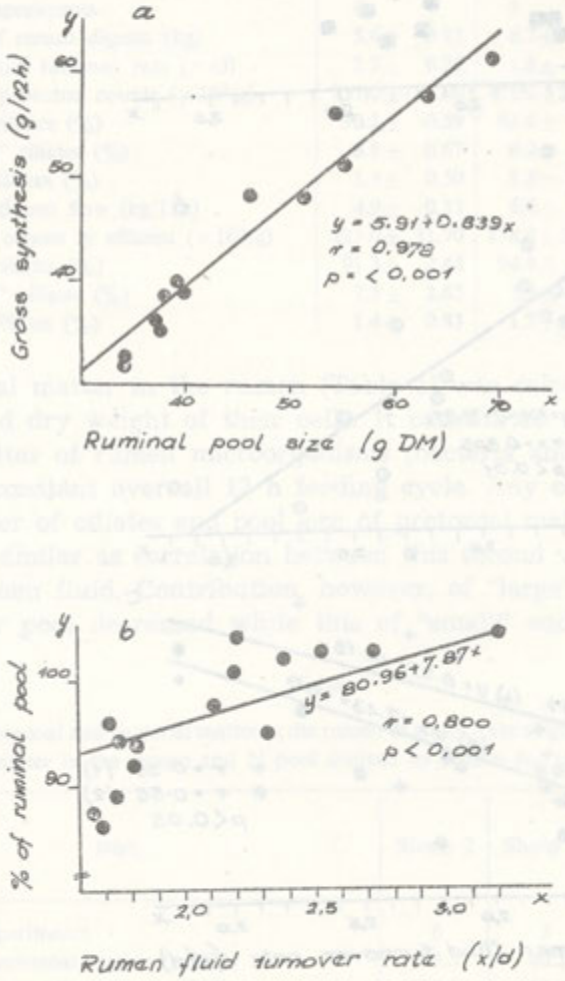

Fig. 2. Straight-line relationship between ruminal pool size of protozoal matter and gross synthesis rate of ciliate matter in the rumen (a) and between efficiency of gross synthesis (expressed as % of the ruminal pool size) and turnover rate of rumen fluid (b)

their number in three growing sheep (Table 3). The calculated gross production (Fig. 2) was equivalent to 90-105% of the ruminal pool size in relation to outflow rate of rumen fluid and contribution of particular ciliate groups to pool of protozoal matter in the rumen. Mean results are given in Table 5 together with data concerning the pool size of N used for protozoal protein synthesis. This pool was equivalent to 26-36% of the dietary N. The rate of gross production of total protozoal matter was tightly correlated with ruminal pool size. The gross synthesis rate of "small" and "medium" ciliates increased and this of "large" ciliates decreased with an increase in turnover rate of rumen fluid (Fig. 3).

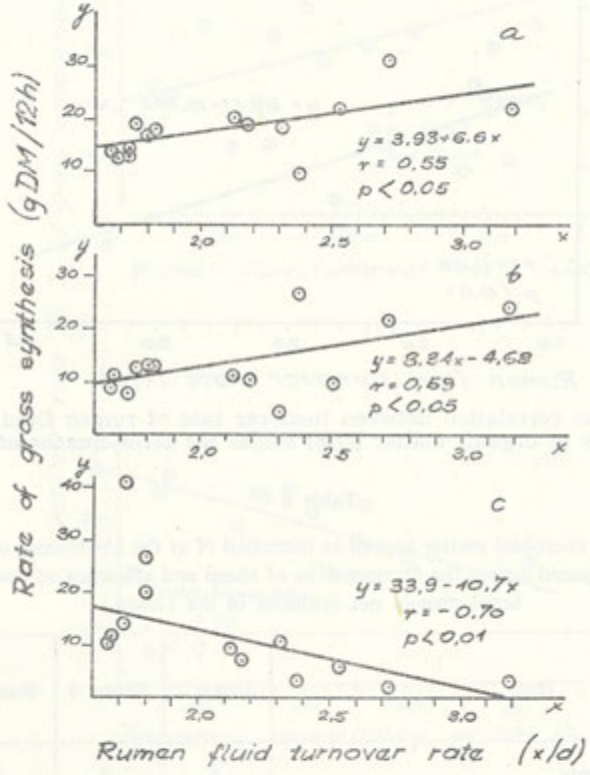

Fig. 3. Straight-line correlation between turnover rate of rumen fluid and rate of gross production of the matter of "small" (a), "medium" (b) and "large" (c) protozoa in the sheep rumen

Dry matter of the digesta leaving the omasum varied from about 195 to 390 g/12 h in relation to an animal and a turnover rate of rumen fluid. Organic matter (OM) constituted 76-90% of this pool and was equivalent to 29-64% of the organic matter of food ration. Thus the apparent digestibility of OM across the forestomachs of sheep va-

ried in range of 36-71% and was negatively correlated with turnover rate of rumen fluid (Fig. 4). Pool size of OM apparently digested in forestomachs of particular animals are presented in Table 6.

Ciliates contributed only to 2.8-6.8% of the pool size the total DM leaving forestomachs of sheep. Mean values are given in Table 6. Ciliates entering abomasum of sheep contributed to 14-39% of the cellular matter synthesized by ciliates in the rumen (see Table 5 and Table 6).

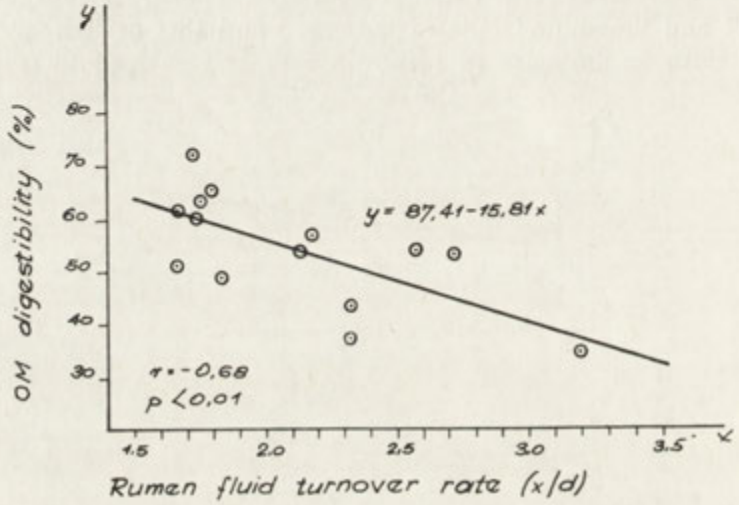

Fig. 4. Straight-line correlation between turnover rate of rumen fluid and apparent digestibility of organic matter (OM) across the forestomachs of sheep

Table 6

Flow of organic and microbial matter as well as microbial N at the abomasum of sheep, organic matter apparently digested across the forestomachs of sheep and efficiency of protozoal and bacterial protein net synthesis in the rumen

Item	Sheep 2	Sheep 4	Sheep 5	Standard error
Number of experiments	6	5	3	
Organic matter flow (g/12h)	235	254	239	15.10
OM apparently digested (g/12h)	293	274	289	12.00
Protozoal matter flow (g DM/12h)	13.5	12.3	11.2	1.07
Protozoal N flow (g/12h)	1.2	1.1	1.0	0.30
Efficiency of protozoal protein synthesis*	4.5	4.4	2.1	0.57
Bacterial matter flow (g DM/12h)	59.5	86.5	45.1	4.28
Bacterial N flow (g/12 h)	4.9	7.1	3.7	0.52
Efficiency of bacterial protein synthesis*	17.6	28.1	12.8	3.09

* — expressed as g N/kg OM apparently digested

This proportion was negatively correlated with efficiency of OM digestion in the forestomachs and positively with the outflow rate of rumen fluid (Fig. 5). Similar relationships were found also in the case of particular groups of ciliates. However, the true outflow rate (g DM/12 h) of "small" and "medium" ciliates increased and this of "large" protozoa decreased with an increase in turnover rate of rumen fluid (Fig. 6).

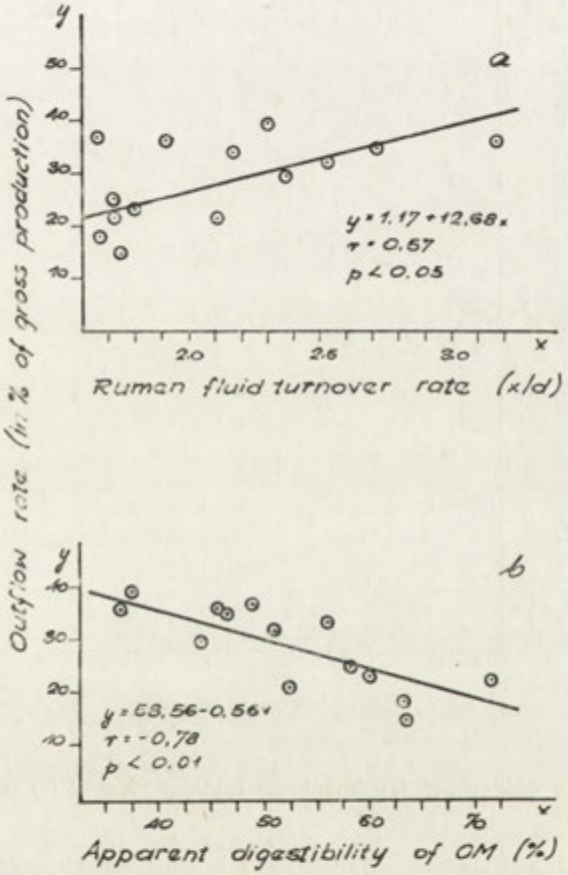

Fig. 5. Relationship between outflow rate of protozoal matter from the forestomachs of sheep (expressed as % of gross synthesis) and both turnover rate of rumen fluid (a) and apparent digestibility of organic matter (OM) across the forestomachs (b)

Bacteria constituted 79-87% of microbial matter entering abomasum of sheep (Table 6). Pool size of bacteria leaving the forestomachs during 12 h after feeding was equivalent to 27-65% of mean ruminal pool size of these micoorganisms in relation to the turnover rate of rumen fluid and efficiency of organic matter digestion in forestomachs (Fig. 7).

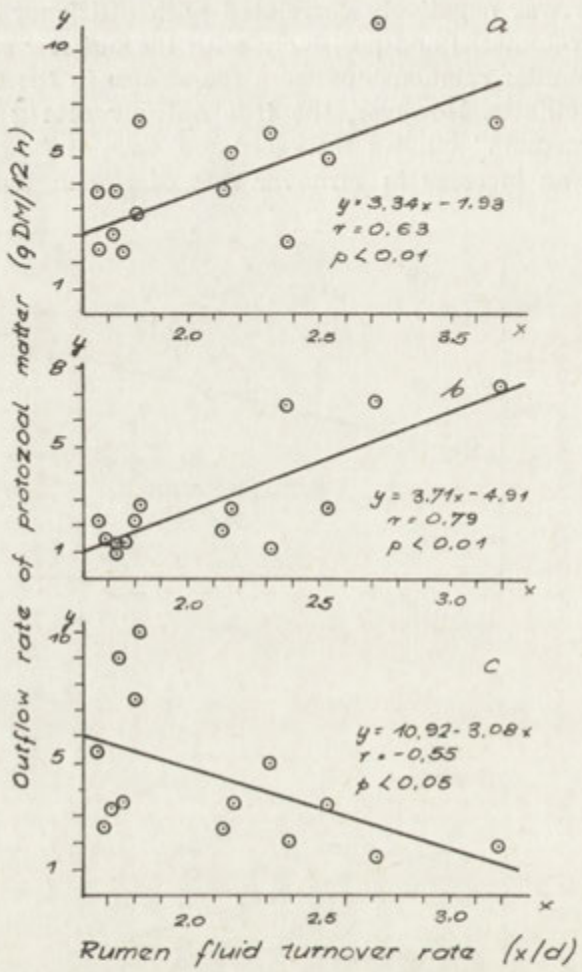

Fig. 6. Straight-line correlation between turnover rate of rumen fluid and outflow rate of the matter of "small" (a) "medium" (b) and "large" (c) protozoa from the forestomachs of sheep

Amino acids of rumen ciliates contributed to 12-23% of microbial amino acids entering abomasum of sheep. Mean pool size of bacterial and protozoal amino acids is presented in Table 7.

Pool size of protozoal matter degraded within the rumen (Table 8) was obtained as a result of substracting of the pool of matter leaving forestomachs from this synthesized at the same time in the rumen. It was equivalent to 58-86% of gross production and increased with an increase in apparent digestibility of OM in forestomachs (Fig. 8). Similar relationship was found regarding to particular groups of ciliates. Degraded cellular matter constituted respectively 72.4 (S.E. 2.16), 78.3 (S.E. 1.0) and

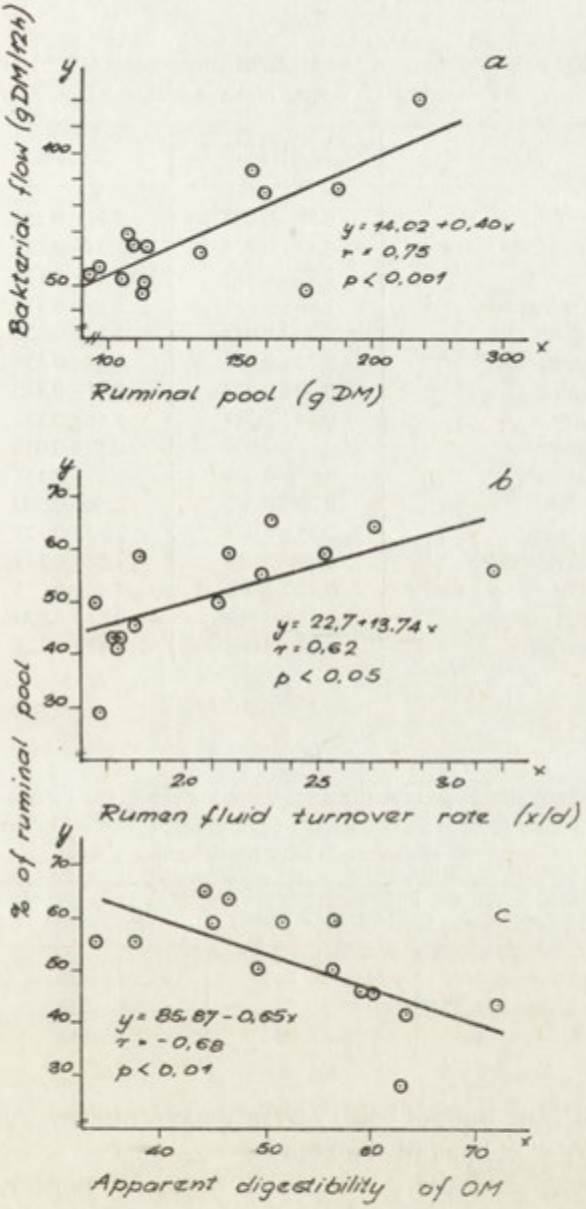

Fig. 7. Straight-line cerrelation between pool size of bacterial matter in the rumen and bacterial flow at the abomasum of sheep, as well as bacterial flow (expressed as % of ruminal pool) and turnover rate of rumen fluid (b) or apparent digestibility of organic matter (OM) across the forestomachs of sheep (c)

Table 7

Daily flow of protozoal and bacterial amino acids (g/12h) at the abomasum of sheep. Mean values ±S.E.

Amino acids	Protozoal	Bacterial
Asparatic acid	0.98±0.083	3.71±0.350
Threonine	0.40±0.029	1.74±0.161
Serine	0.34±0.029	1.46±0.136
Glutamic acid	1.06±0.086	3.56±0.318
Proline	0.26±0.027	1.18±0.328
Glycine	0.32±0.031	1.62±0.154
Alanine	0.30±0.027	2.71±0.445
Valine	0.34±0.029	1.46±0.138
Methionine	0.10±0.009	0.37±0.038
Isoleucine	0.47±0.040	2.13±0.157
Leucine	0.60±0.051	2.54±0.240
Tyrosine	0.25±0.021	1.31±0.125
Phenyloalanine	0.39±0.034	1.56±0.144
Lysine	0.47±0.040	2.48±0.235
Histidine	0.11±0.009	0.51±0.090
Arginine	0.34±0.048	1.47±0.125

Table 8

Pool size of protozoal matter degraded across the forestomachs (g/12 h) and pool size of protozoal N recycled in the rumen of sheep (g/12 h). Mean values ±S.E.

Animal	Degraded matter	Recycled N
Sheep 2	39.3±3.00	3.6±0.25
Sheep 4	25.4±2.24	2.3±0.22
Sheep 5	28.2±1.61	2.6±0.13

56.8 (S.E. 4.46)% of the pool size of matter synthesized by "small", "medium" and "large" ciliates in the rumen.

From N pool used for synthesis of protozoal matter, i.e. protozoal protein only 1-1.2 g contributed to protein leaving the forestomachs during 12 h after feeding (Table 6) while the rest was recycled in the rumen (Table 8). Protozoal N constituted 13-21% of the microbial N entering abomasum of sheep and efficiency of protozoal protein net synthesis was 4-6 times lower than the bacterial (Table 6). It was tightly related to digestion rate of OM in the rumen and to the turnover rate of rumen fluid (Fig. 9).

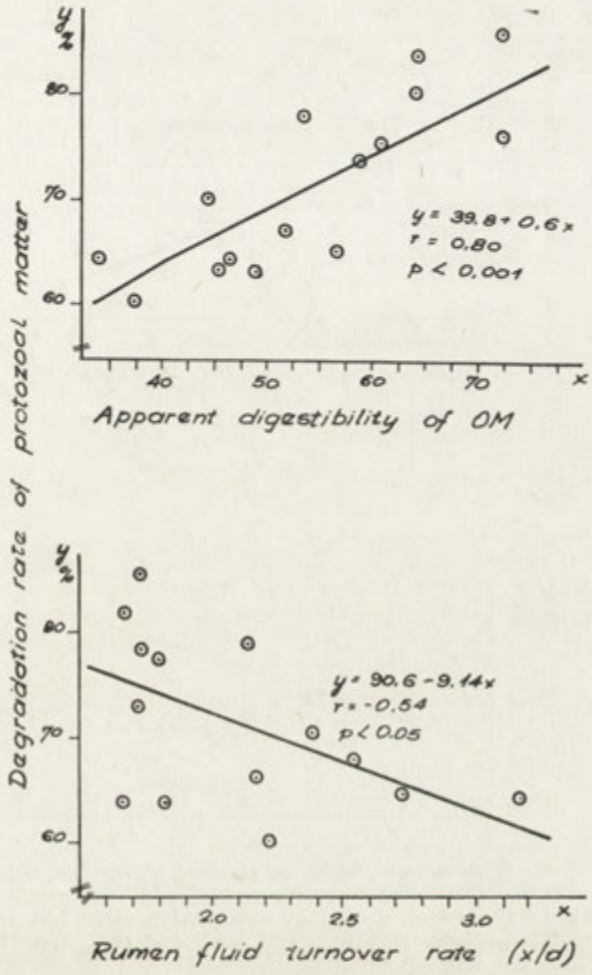

Fig. 8. Straight-line correlation between pool size of protozoal matter degraded in the rumen (expressed in % of gross synthesis) and both apparent digestibility of organic matter (OM) across the forestomachs (a) and turnover rate of rumen fluid (b)

Discussion

The changes in the matter of rumen ciliates have been described in the report. They were calculated from cellular dry matter of protozoa, their chemical composition as well as number and growth rate. Therefore a validity of the presented results depended on accuracy of direct measurements performed during the studies.

The ciliates were routinely counted using Fuchs-Rosenthal counting chamber, however, the dilution counting method (M i c h a ł o w s k i 1975)

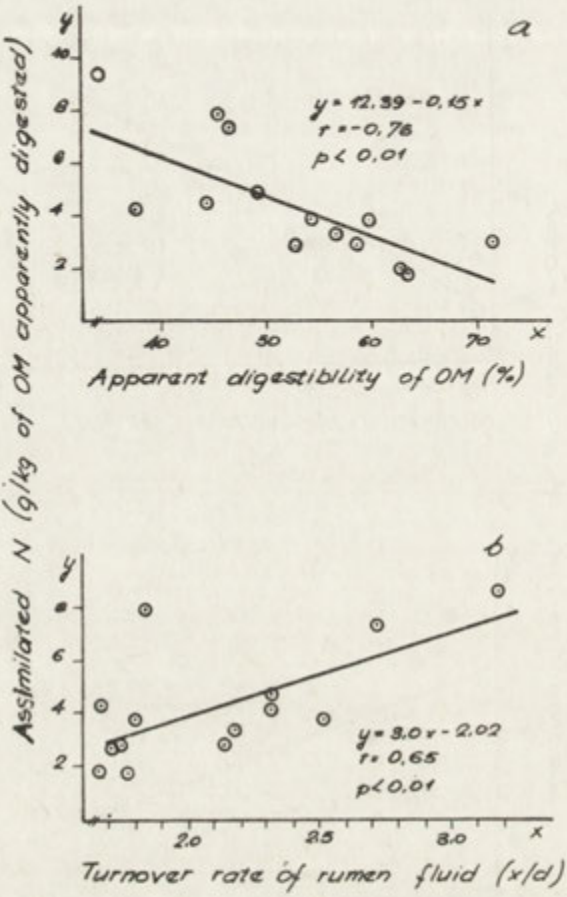

Fig. 9. Relationship between efficiency of protozoal protein net synthesis (expressed as g N/kg OM apparently digested) and both apparent digestibility of organic matter OM across the forestomachs of sheep (a) and turnover rate of rumen fluid (b)

was also used if necessary, especially when dry matter of single cells and growth rate in ciliate number were estimated. Thus it seems that results concerning protozoal biomass and its synthesis are of sufficient accuracy.

Unfortunately the flow at the abomasum was calculated from concentration of one marker only (CrEDTA). The obtained results however, are comparable with these calculated on the basis of two markers (Meyer et al. 1986). In fact only one marker was also used for measuring of duodenal flow by Harrisin et al. (1979), Mercer et al. (1982), Leng (1982), Siddons et al. (1982) and others.

Rumen content volume before feeding was computed from concentration of CrEDTA in rumen fluid as described previously (Michałow-

ski et al. 1986). A decrease, however, in concentration of this marker after feeding seems to be representative of increase in rumen fluid outflow rate rather than of an increase in rumen fluid volume (Warner and Stacy 1968). Thus rumen content volume after feeding was calculated from the pool size of dry matter as described in "Material and Methods" and obtained results are in good agreement with results of other authors (Meyer et al. 1965).

Performed estimations of dry weight of ciliate cells showed huge differences between protozoa species and they are in good agreement with differences between dimensions of ciliates (Dogiel 1927, Grain 1966) as well as between their volume (Harmeyer and Hill 1964, Hungate et al. 1971, Clarke et al. 1982). Therefore biomass of ciliates in the rumen of sheep calculated from cellular dry matter (Table 5) was similar to this estimated from volume of the cells (Hungate et al. 1971, Clarke et al. 1982) if number of protozoa did not differ significantly.

Contribution of ciliates to microbial mass in the rumen was only of 18-32% comparing with 50 (Harrison et al. 1979) or about 80% (Coleman 1975). In general the estimated proportions are in agreement with the results revived by Harrison and Mc Allen (1980) and concerning the relationship between ciliate concentration in the rumen fluid and their contribution to microbial mass. Pool size of protozoal metter, however, did not depend on the total number of protozoa in present experiments. This discrepancy was the result of quite different contribution of particular groups of ciliates to total protozoa counts (Table 4) and to protozoal biomass (Fig. 1). Total protozoa counts was affected by the changes in number of the "small" ciliates which constituted 88-94% of all ciliates in the rumen. On the contrary the changes in number of "large" ciliates had rather small (if any) effect on the total protozoa counts (Table 4). These changes, however, markedly influenced the total protozoal biomass due to high cellular dry wight of large ciliates (Table 1). Therefore an increase in total ciliate number was not accompained by an increase in pool size of protozoal matter because a decrease in the number of "large" ciliates was observed there (Fig. 1). The obtained results suggest that total protozoa counts seems not to be a satisfactory gauge of the pool size of ciliate matter in the rumen and that specific counts of ciliate should be also considered.

Doubling time of population varied in a range of 8.2-10.3 h (Table 3). These results are in good agreement with findings of Warner (1962), Potter and Dehority (1973), Senaud et al. (1973) and Steinhour et al. (1982). However, Singh et al. (1974), Leng (1982), Leng et al. (1981, 1984, 1986) and Ffoulkes and Leng (1988) have found

the doubling time of ciliates as long as 11-21 h. According to this the rate of gross synthesis of protozoal matter was as high as 94-99% of the mean ruminal pool size/12 h when calculated from the growth rate presented in the paper (Table 5) and 90-150%/24 h when calculated by the authors cited from a dilution rate of an isotope marker. Thus the discussed data may suggest that a rate of synthesis of cellular mater of ciliates depended on the calculation methods. However it is possible and presumabely it is true, that synthesis rate of the cellular matter of ciliates in the rumen is affected by some factors. It is also possible that one of these factors is the diet and/or feeding regime. The similar growth rate of ciliates irrespective to different food doses given to animals proportionally to their live weight (Table 3) seems to confirm such possibility. The appropriate studies are, however, needed. Leng et al. (1984) found that the high turnover rate of rumen fluid was a factor stimulating production rate. Results obtained in present studies confirm these findings in the case of "small" and "medium" ciliates only. In fact, the production was closely related to pool size of ciliate matter in the rumen and this last was a resultant of the formation rate of new cells of ciliates, their outflow and degradation rate in the rumen.

Protozoal pool size in the rumen was relatively constant in spite to a distinct prevalence of production over the outflow (Table 5 and 6). Thus this difference was considered as a pool of ciliates degraded within the forestomachs. Obtained results suggest that degradation was a permanent process and that within the time when 1 g of cellular matter was synthesized as much as 0.6-0.9 g was decomposed making the ruminal pool size unchanged. Therefore it was possible to calculate the gross production from the mean pool size of protozoal matter in the rumen. Degradation rate of ciliates in the rumen calculated in this report was higher comparing to findings of H u n g a t e et al. (1971) and Leng (1982). The production rate was, however, also higher.

One of the factors promoting degradation of ciliates seems to be a long residence time of these organisms in the rumen (L e n g et al. 1981, M i c h a ł o w s k i et al. 1986). Results presented here showed that a decrease in the removal rate of digesta from the forestomachs enhanced not only digestion rate of organic matter (Fig. 3) but also decomposition of ciliate cells (Fig. 8) and this degradation concerned especially to "small" and „medium" ciliates. This enlarged degradation rate at low removal of digesta could be a concequence of prolonged rumination time as well as prolonged bacteria action against the protozoa. Numerous microscopial observations (unpublished) have shoved that bacteria attack not only death protozoa but also impaired or/and weakened ciliates. Such protozoa should be present all the time in the rumen as a result of chewing

the food bolus by the host as well as of violent knocks of ciliates against rough food particels when these organisms are floated with the liquid stream after contraction of rumen wall. Hence an increase in the outflow rate of rumen fluid enhanced the pool size of ciliates leaving the forestomachs and reduced on this way a degradation of protozoal matter in the rumen. On the some way it improved the net efficiency of protozoal protein formation (Fig. 9). It is also possible that „large" ciliates are more resistant to mentioned knocks since they posses more thight cuticula which causes their lower degradation rate comparing to other ciliates.

Contribution of ciliates to N utilization was calculated from the results of estimation of total nitrogen as well as amino acids contents of their matter. Proportion of N in microbial dry matter (Table 1) are similar to these of Hutton et al. (1971), Hungate et al. (1971), Hagemeister (1975) and Cockburn and Williams (1984). However, the contribution of N to dry mass of rumen microorganisms and especially of protozoa can be periodically lowered by the increasing proportions of other components, e.g. of storage carbohydrates and food particles. This fact explains a lower proportion of N in protozoal matter (Czerkawski 1976, Michałowski 1979) comparing to the results of present report. High proportion of total N in protozoal matter was accompanied by proportionally high amino acid N level (Table 2). In general the results concerning amino acid composition of ciliates and bacteria are in good agreement with findings of Hoeller and Harmeyer (1964), Purser and Buechler (1966) and Cockburn and Williams (1984). The higher proportion of lysine in protozoa than in bacteria was, however, not found (Table 2) perhaps due to different preparation or analysis procedure.

Obtained results showed that ciliates used as much as 3.5-4.8 g N for synthesis of their cellular matter per 12 h. However, 55-86% was turned over in the rumen. On the contrary Harison and Mc Allen (1980) concluded that only few if any ciliates are recycled in the rumen when dilution rate of rumen fluid was not lower than 1.8 per day. They based, however on the assumption that the mean division time of ciliates was 24 h while in present studies it was not longer than 10.3 h (Table 3).

Due to high recycling of protozoal N the role of ciliates as a source of N for the host was diminished because only 1-1.2 g protozoal N was entering abomasum during 12 h (Table 6) and it constituted only 13-21% of the microbial N. These results are in very good agreement with findings of John and Ulyatt (1984) who have calculated a pool size of protozoal N entering lower gut of sheep from concentration of phosphatidil choline in appropriate digesta and in ciliates. The results of other

authors ranged, however, from 0.14-0.44 (Weller and Pilgrim 1974) to 5.2 g/day (Meyer et al. 1986). Some of these results are uncertain since they were calculated from concentration of AEPA in duodenal digesta. Thus further studies seem to be still needed, especially with respect to the diet offered to ruminants.

In general the contribution of ciliates to microbial amino acids entering duodenum was equivalent to contribution of protozoal N to microbial N (see above). Unfortunatelly these results confirmed the opinion that protozoa are of small importance as a source of amino acids for the host (Weller and Pilgrim 1974).

Efficiency of protozoal protein net synthesis varied in range of 1.4-8.1 g N/kg OM apparently digested in the forestomachs of sheep (Fig. 9). These results are comparable with findings of John and Ulyatt (1984). It was faund in experiments performed parallely on the same animals that the production rate of volatile fatty acids (VFA) varied in range of 1.42-1.84 mmol per gram of protozoal DM per hour while total microbial production was 2.32-2.91 mol/12 h (Michałowski 1987). Thus it was possible to calculate an approximate contribution of ciliates to pool size of organic matter digested in the forestomachs using a combination of the results cited above and from the presented report. The calculation was based on an assumption that to ratio of VFA produced/OM apparently digested (mol/kg) is constant one (Gray et al. 1967, Czerkawski 1986). Obtained results showed that as much as 31-36% or 84-110 g of organic matter in the rumen was digested by protozoa. Hence the corrected efficiency of the ciliate protein net synthesis was 10.5-13.1 g N/kg OM digested by protozoa while improved efficiency of bacterial protein formation was 19-37 g N/kg OM digested by bacteria, i.e. 2-3 times higher. One of the causes of the lower efficiency of protozoal net production seems to be the high rate of ciliate degradation in the rumen due to their selective retention in this compartment (Michałowski et. al. 1986). Another factor seems to be a presumably high requirement of ciliates for maintenance energy (Harmeyer 1986). The rumen protozoa are of large dimensions, high cell organisation and of high locomotor cativity as compared to the bacteria. Thus the costs they must pay for their own existence in the rumen ecosystem are presumably much higher than these of bacteria.

Data presented in the report showed that contribution of ciliates to pool of N as well as pool of organic matter utilized in the rumen tended to be somewhat higher than their contribution to microbial biomass. These results confirm the opinion about an important role of protozoa in fermentation of food constituents (Veira 1986). A high contribution of ciliates to fermentation processes may be, however, one of the causes of

insufficiency in N assimilation by faunated ruminants (Veira 1986) since the majority of the synthesized protozoal protein is degraded in the forestomachs. Thus a minor supply of microbial N to the lower gut of faunated animals as compared to the ciliate-free (Lindsay and Hogan 1972, Demeyer and Van Nevel 1979, Veira et al. 1983) seems to be a result of not only the utilization of bacterial protein by protozoa but also a consequence of recycling of the majority of N used by ciliates for their cellular matter synthesis in the rumen.

Conclusions

A main bulk of microbial matter in the rumen of sheep fed hay-concentrate diet constituted bacteria. A pool size of ciliate matter was not related to total count of protozoa and was visibly affected by organisms of large cell dimensions constituting less than 2% of the total protozoa number.

Formation of the new cells of ciliates took place over all the 12 h feeding cycle while the efficiency of gross synthesis made possible a doubling of the pool of protozoal matter in the rumen within 12 h. Contribution of particular groups of ciliates to total protozoa counts had a restricted effect on the gross synthesis of the ciliate matter.

The majority of the synthesized protozoal matter was degraded within forestomachs presumably due to selective retention of ciliates in the rumen since only 14-39% of protozoal matter left the rumen ecosystem in the form of intact cells. High degradation rate of ciliates in the rumen resulted in low efficiency of net synthesis of the protozoal protein and recycling in the rumen of the majority of N used by ciliates for synthesis of the cellular matter.

A factor influencing the turnover of protozoal matter was the outflow rate of rumen digesta. An increase in the outflow rate enhanced the removal of intact ciliates from the forestomachs and diminished the pool of protozoal matter turned over in the rumen.

Activity of ciliates in utilization of N and organic matter for the growth was presumably not lower than this of bacteria. It is possible that contribution of ciliates to the pool size of the nutrients converted in the rumen is of the range of their contribution to the pool of microbial matter in this compartment.

High turnover rate of protozoal matter in the rumen diminished presumably a role of protozoa as an amino acid source for the host and might

negatively influence the efficiency of utilization of the food by ruminants for their growth.

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REFERENCES

Abou Akkada A. R. and El Shazly K. 1976: Non protein nitrogen utilization and microbial synthesis in the rumen. In: Tracer Studies on Non-Protein-Nitrogen for Ruminants, Alexandria I.E.A.E., Vol. 3, 49-56.

Akin D. E. 1986: Chemical and biological structure in plants as related to microbial degradation of forage cell walls. In: Control of Digestion and Metabolism in Ruminants. (Eds. L. P. Milligan, W. L. Grovum and A. Dobson). A Reston Book, Prentice-Hall, Englewood Cliffs. New Jersey, 139-157.

Cockburn J. E. and Williams A. P. 1984: The simultaneous estimation of the amounts of protozoal, bacterial and dietary nitrogen entering the duodenum of steers. Br. J. Nutr., 51, 111-132.

Clarke R.T.J., Ulyatt M. J. and John A. 1982: Variation in numbers and mass of ciliate protozoa in the rumen of sheep fed schaffed alfalfa (Medicago sativa). Appl. Environ. Microbiol., 43, 1201-1204.

Coleman G. S. 1975: The interrelationship between rumen ciliate protozoa and bacteria. In: Digestion and Metabolism in the Ruminant. (Eds. I. W. Mc Donald and A. C. I. Warner). The University of New England Publishing Unit, Armidale, 149-164.

Coleman G. S. 1983: Hydrolysis of fraction 1 leaf protein and casein by rumen entodiniomorphid protozoa. J. Appl. Bacteriol., 55, 111-118.

Coleman G. S. 1985: The cellulase content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. J. Agric. Sci. Camb., 104, 349-360.

Coleman G. S., Davies J. I. and Cash M. A. 1972: The cultivation of the rumen ciliates *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum* in vitro. J. Gen. Microbiol., 73, 509-521.

Czerkawski J. W. 1974: Methods for determining 2, 6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. J. Sci. Fd. Agric., 25, 45-55.

Czerkawski J. W. 1986: An Introduction to rumen Studies. Pergamon Press, Oxford, New York, Toronto, Sydney, Frankfurt.

Demeyer D. I. 1981: Rumen microbes and digestion of plant cell walls. Agr. Environ., 6, 295-337.

Demeyer D. I. and Van Nevel C. J. 1979: Effect of defaunation on the metabolism of rumen microorganisms. Br. J. Nutr., 42, 515-524.

Dogiel V. A. 1927: Monographie der Familie Ophryoscolecidae. Arch. Protistenkd., 59, 1-288.

Egan A. R. 1974: Protein-energy relationships in the digestion products of sheep fed on herbage diets differing in digestibility and nitrogen concentration. Aust. J. Agric. Res., 25, 613-630.

Ffoulkes D. and Leng R. A. 1988: Dynamic of rumen protozoa in the rumen of cattle. Br. J. Nutr., 59, 429-436.

Forsberg C. W., Lovelock L. K. A., Krumholz L. and Buchanan-Smith J. G. 1984: Protease activities of rumen protozoa. Appl. Environ. Microbiol., 47, 101-110.

Grain J. 1966: Etude cytologique de quelques ciliés holotrichs endocommensaux des ruminants et des equides. Protistologica 2, 5-141.

Gray F. V., Weller R. A., Pilgrim A. F. and Jones G. B. 1967: Rates of production of volatile fatty acids in the rumen. V. Evaluation of fodders in terms of volatile fatty acid production in the rumen of sheep. Aust. J. Agric. Res., 18: 625-634.

Hagemeister H. 1975: Messungen von Protozoen-Eiweiss in Darm von Wiederkäuern mit Hilfe von 2-Aminoäthyl-phosphonischer Säure AAP und seine Bedeutung für die Einweissversorgung. Kiel. Milchwirtsch. Forschungsber., 27, 347-354.

Harmeyer J. 1986: Energy expenditures of rumen microbes for maintenance. Arch. Anim. Nutr., 36, 143-148.

Harmeyer, J. and Hill H. 1964: Das Protozoenvolumen im Panseninhalt bei Ziege und Guanako. Zentralbt. Veterinaermed. Reihe A 11, 493-501.

Harrison D. G., Beever D. E. and Osbourn D. F. 1979: The contribution of protozoa to the protein entering the duodenum of sheep. Br. J. Nutr., 41, 521-527.

Harrison D. G. and Mc Allen A. B. 1980. Factors affecting microbial growth yields in the reticulo-rumen. In: Digestive Physiology and Metabolism in Ruminants. (Eds. Y. Ruckenbusch and P. Thivend). MTP Press Limited, Lancaster, 205-226.

Hoeller H. and Harmeyer J. 1964: Nitrogen and amino acid content of rumen protozoa. Zentr. Vet. Med., Reihe A 11, 244-252.

Hungate R. E. 1986: Challenges in rumen microbiology. In: Control of Digestion and Metabolism in Ruminant. (Eds. L. P. Milligan, W. L. Grovum and A. Dobson). A Reston Book, Prentice-Hall Englewood Cliffs, New Jersey, 531-539.

Hungate R. E., Reichl J. and Prins R. 1971: Parameters of rumen fermentation in a continuously fed sheep: Evidence of a microbial rumination pool. Appl. Microbiol., 22, 1104-1113.

Hutton K., Bailey F. J. and Annison E. F. 1971: Measurement of the bacterial nitrogen entering the duodenum of the ruminant using diaminopimelic acid as a marker. Br. J. Nutr., 25, 165-173.

John A. and Ulyatt M. 1984: Measurement of protozoa using phosphatidyl choline, and of bacteria, using nucleic acids, in the duodenal digesta of sheep fed chaffed lucerne hay (*Medicago sativa* L..) diets. J. Agric. Sci. Camb., 102, 33-44.

Jouany J. P. and Senaud J. 1979: Role of rumen protozoa in the digestion of food cellulosic materials. Ann. Rech. Vet., 10, 261-263.

Kayouli C., Demeyer D. I., Van Nevel C. J. and Dendoowen R. J. 1984: Effect of defaunation on straw digestion in sacco and on particle retention in the rumen. Animal. Feed. Sci. Technol., 10, 165-172.

Leng R. A. 1982: Dynamics of protozoa in the rumen of sheep. Br. J. Nutr., 48, 399-415.

Leng R. A., Dellow D. and Waghorn G. 1986: Dynamics of large ciliate protozoa in the rumen of cattle fed on diets of freshly cut grass. Br. J. Nutr., 56, 455-462.

Leng R. A., Gill M., Kempton T. J., Rowe J. B., Nolan J. V., Stachiw S. J. and Preston T. R. 1981: Kinetics of large ciliate protozoa in the rumen of cattle given sugar cane diets. Br. J. Nutr., 46, 371-384.

Leng R. A., Nolan J. V., Cumming G., Edwards S. R. and Graham C. A. 1984: The effects of monensin on the pool size and turnover rate of protozoa in the rumen of sheep. J. Agric. Sci. Camb., 102, 609-613.

Lindsay J. R. and Hogan J. P. 1972: Digestion of two legumes and rumen bacterial growth in defaunated sheep. Aust. J. Agric. Res., 23, 321-331.

Meyer J. H., Kromann R. and Garrett W. N. 1965: Digestion (Influence of roughage preparation). In: Physiology of Digestion in the Ruminants. (Eds. R. W. Dougherty, R. S. Allen, W. Bouroughs, N. L. Jacobson and A. D. Mc Gilliard). Butterworths, London, 262-271.

Meyer J. H. F., Van der Walt S. I. and Schwartz M. H. 1986: The influence of diet and protozoal numbers on the breakdown and synthesis of protein in the rumen of sheep. J. Anim. Sci., 62, 509-520.

Mercer J. R., Allen S. A. and Miller E. L. 1980: Rumen bacterial protein synthesis and proportion of dietary protein escaping degradation in the rumen of sheep. Br. J. Nutr. 43, 421-433.

Michałowski T. 1975: Effect of different diets on the diurnal concentration of ciliate protozoa in the rumen of water buffalo. J. Agric. Sci. Camb. 85, 145-150.

Michałowski T. 1979: Effect of feeding frequency on the diurnal changes in microbial protein, volatile fatty acids and ammonia contents of the rumen of sheep. J. Agric. Sci. Camb., 93, 67-70.
Michałowski T. 1987: The volatile fatty acids production by ciliate protozoa in the rumen of sheep. Acta Protozool., 26, 335-345.
Michałowski T., Harmeyer J. and Breves G. 1986: The passage of protozoa from the reticulo-rumen through the omasum of sheep. Br. J. Nutr., 56, 625-634.
Potter E. L. and Dehority B. A. 1973: Effects of changes in feed level, starvation and level of feed after starvation upon the concentration of rumen protozoa in the ovine. Appl. Microbiol., 26, 692-698.
Purser D. B. and Buechler S. M. 1966: Amino acid composition of rumen microorganisms. J. Dairy Sci., 49, 81-84.
Ruszczyc Z. 1970: Metodyka doświadczeń zootechnicznych. PWRiL, Warszawa.
Senaud J., Jouany J., Grain J.-P. and Puytorac C. 1973: Dynamique d'une population de *Polyplastron multivesiculatum* (Cilie, Oligotriche) en equilibre dans le rumen de mouton. Comp. Rend. Acad. Aci., Paris, 277, 197-200.
Siddons R. C., Beever D. E. and Nolan J. V. 1982: A comparison of methods for the estimation of microbial nitrogen in duodenal digesta of sheep. Br. J. Nutr., 48, 377-389.
Singh U. B., Varma A., Verma D. N. and Runjhan S. K. 1974: Measurement in vivo of the rate of production of protozoa in the rumen. J. Dairy Res., 41, 299-303.
Steinhour W. D., Stokes M. R., Clark J. H., Rogers J. A. and Davis C. L. 1982: Estimation of the proportion of non-ammonia-nitrogen reaching the lower gut of the ruminant derived from bacterial and protozoal nitrogen. Br. J. Nutr., 48, 417-431.
Veira D. M. 1986: The role of ciliate protozoa in nutrition of the ruminant. J. Anim. Sci., 63, 1547-1560.
Veira D. M., Ivan M. and Jui P. Y. 1983. Rumen ciliate protozoa: Effects on digestion in the stomach of sheep. J. Dairy Sci., 66, 1015-1021.
Warner A. C. I. 1962: Some factors influencing the rumen microbial population. J. Gen. Microbiol., 28, 129-146.
Warner A. C. I. and Stacy B. D. 1968: The fate of water in the rumen. 2. Water balances through the feeding cycle in sheep. Br. J. Nutr., 22, 389-410.
Weller R. A. and Pilgrim A. F. 1974: Passage of protozoa and volatile fatty acids from the rumen of the sheep and from continuous in vitro fermentation system. Br. J. Nutr., 32, 341-351.
Whitelow F. G., Eadie J. M., Bruce L. A. and Shand W. J. 1984: Microbial protein synthesis in cattle given roughage-concentrate and all-concentrate diets: the use of 2,6-diaminopimelic acid and 2-aminoethylphosphonic acid and ^{35}S as markers. Br. J. Nutr., 52, 249-260.
Williams A. G., Withers S. E. and Coleman G. S. 1984: Glycoside hydrolases of rumen bacteria and protozoa. Curr. Microbiol., 10, 287-294.

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A Functional Study of the Dexamethason e-Induced Steroid Receptor in *Tetrahymena*

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Synopsis. Tetrahymena doesn't contain steroid receptors, however these develop in it after steroid treatment. This was demonstrated earlier by receptor-kinetic analysis. Presently a functional index, PAS-positivity was studied. The PAS positivity of the cells decreases under the effect of the first encounter with the ethanol solved form of dexamethasone, however increased significantly in the case of the second treatment, indicating the presence (development) of receptor.

Although the hormone receptors are characteristic of higher organisms, similar structures also occur in invertebrates and unicellulars (Csaba 1980, 1985, 1986). Specific insulin receptors were demonstrated in *Neurospora crassa* (Fawell et al. 1988, Fawell and Lenard 1988, McKenzie et al., 1988) and specific appearing steroid receptors in *Saccharomyces cerevisiae*, *Candida albicans* and *Trichomonas vaginalis* (Ford et al. 1987, Feldman et al. 1982, Loose et al. 1981). We reported earlier that the unicellular *Tetrahymena*, which is able to respond to several polypeptide and amino acid hormones, had not a detectable steroid receptor at primary interaction with a steroid hormone, but did form a specific binding site for the steroid on lasting exposure (Csaba et al. 1985). In the present study we investigated the functional expression of the induced steroid binding site by comparison to the response of not previously treated (imprinted) *Tetrahymena* cells.

Material and Methods

Tetrahymena pyriformis GL cells cultured in yeast extract containing Bactotryptone medium at 28°C were treated in the logarithmic phase of growth with 10^{-6}M dexamethasone (Serva, Heidelberg) for 72h, returned to plain medium for 48h, and re-exposed to dexamethasone for 24h. Since dexamethasone was dissolved in presence of 0.07 per cent ethanol, an ethanol control series, too, was set up in addition to the absolute (untreated) control cultures. Cells fixed 24h after the first and the second dexamethasone treatment were treated with PAS reagent for a quantitative cytophotometric assay. A Zeiss cytophotometer, which was connected with a HP41 CX calculator for data processing, was used. Twenty cells were assayed in each group and each assay was performed in three replicates, thus each column of the attached diagram (Fig. 1.) represents a mean value for 60 cells.

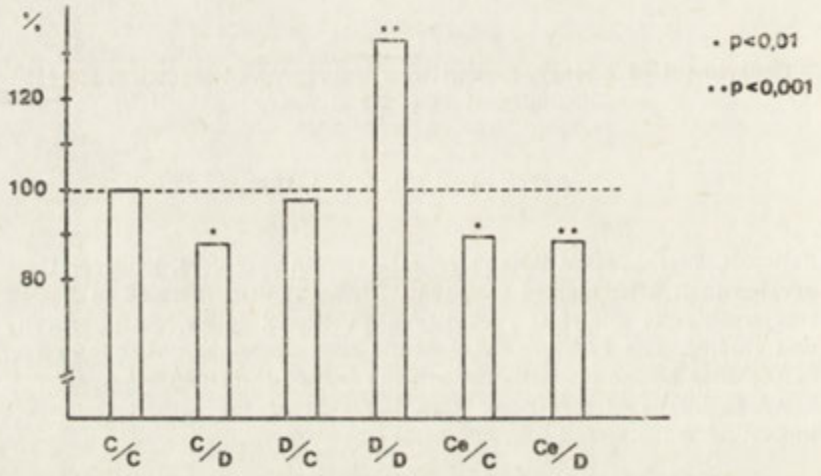

Fig. 1. PAS positivity of *Tetrahymena* cells evaluated by quantitative cytophotometry related to the double untreated control (C/C) as 100. C — untreated, D — dexamethasone treated, Ce — ethanol control

Results and Discussion

As shown in earlier studies (Csaba and Kovács 1979), the hormones influence the glucose metabolism not only in higher organisms, but also in unicellular ones. Thus the PAS reaction, which detects the intracellular accumulation of glycogen, can serve as indicator of glycocorticoid action. In the present experiment the PAS-positivity of *Tetrahymena* was significantly decreased after primary interaction with dexamethasone but since the cells of the ethanol control series showed a similar decrease, this phenomenon was clearly unrelated to hormone action. However, the second exposure to dexamethasone resulted in a biologically

and statistically equally significant increase in PAS-positivity over the control. It follows that the induced steroid receptor, whose formation had been substantiated earlier by receptor kinetic evidence (Csaba and Inczefi-Gonda 1989, Csaba et al. 1985) was functioning in *Tetrahymena* as a genuine receptor, also in respect of mediation of the signal (hormone) molecule. This did, of course, presuppose the collaboration of the signal mediation structures involved in the transfer of information from the receptor to the intracellular functional unit. This is not surprising, if it is taken into consideration that the human oestrogen receptor is able to precipitate the adequate function also after transplantation into yeast (Metzger et al. 1988) cells.

As the cells had been studied certain time after treatment, many new generation developed. Thus the probability of the cell presence of the originally imprinted generation at the time of the assay was as low as 0.097 per cent. It follows that practically all cells involved in reexposure to dexamethasone belonged to a progeny generation and those assayed after reexposure also represented progeny cells. The fact that the latter still showed indications of an increased synthesis of storage of glycogen 24 h after dexamethasone supports the implication of a durable receptor level action of the hormone.

REFERENCES

Csaba G. 1980: Phylogeny and ontogeny of hormone receptors: the selection theory of receptor formation and hormonal imprinting. Biol. Rev. (Cambridge) 55, 47-63.

Csaba G. 1985: The unicellular *Tetrahymena* as a model cell for receptor research. Int. Rev. Cytol. 95, 327-377.

Csaba G. 1986: Why do hormone receptors arise? Experientia 42, 715-718.

Csaba G. and Kovács P. 1979: Complex cytophotometric analysis of hormone-induced alterations in the glucose utilisation of *Tetrahymena*. Endokrinologie 73, 116-119.

Csaba G. and Inczefi-Gonda A. 1989: Specificity of dexamethasone-induced steroid receptor in *Tetrahymena*. Experientia 45, 174-175.

Csaba G, Inczefi-Gonda Á. and Fehér T. 1985: Induction of steroid binding sites (receptors) and presence of steroid hormones in the unicellular *Tetrahymena pyriformis*. Comp. Biochem. Physiol. 82A, 567-570.

Fawell S. E., Mc Kenzie M. A., Greenfield J. J., Adebodun F., Jordan F. and Lenard J. 1988: Stimulation by mammalian insulin of glycogen metabolism in a wall-less strain of *Neurospora crassa*. Endocrinology 122, 518-523.

Fawell S. E. and Lenard J. 1988: A specific insulin receptor and tyrosine kinase activity in the membranes of *Neurospora crassa*. Biochem. Biophys. Res. Comm. 155, 59-65.

Feldman D., Do Y., Burshell A., Stathis P. and Loose D. S. 1982: An estrogen binding protein and endogeneous ligand in *Saccharomyces cerevisiae*. Science 218-298.

Ford L. C., Hammil H. A., DeLange R. J., Bruckner D. A., Suzuki-Chavez F., Mickus K. C. and Lebherz T. B. 1987: Determination of estrogen and androgen receptor in *Trichomonas vaginalis* and the effects of antihormones. Am J. Obstetr. Gynecol. 156, 1119-1121.

Loose D. S., Schurman D. and Feldman D. 1981: A corticosterone binding protein and endogenous ligand in *C. albicans* indicating a possible steroid receptor. Nature 293, 477-479.

McKenzie M. A., Fawell S. E., Cha M. and Lenard J. 1988: Effects of mammalian insulin on metabolism, growth and morphology of a wall-less strain of *Neurospora crassa*. Endocrinology 122, 511-517.

Metzger R. D., White J. H. and Chambon P. 1988: The human oestrogen receptor functions in yeast. Nature 334, 31-36.

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Effects of Organophosphorus Insecticide Phosphamidon on Ciliate Protozoan *Tetrahymena pyriformis*

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Synopsis. This paper describes effects of phosphamidon on the cell population growth and cell dimensions of *Tetrahymena pyriformis*. Phosphamidon at a concentration of 250 ppm was lethal to *Tetrahymena* on the fifth day of treatment. Lower concentrations (0.5-10 ppm) stimulated growth for the first few days and then were slightly inhibitory. At 50 and 100 ppm phosphamidon markedly inhibited growth and the percent inhibition ranged from 63-90 on the fifth day. The changes observed after treatment with 50 and 100 ppm were reversible. The same dosage affected the cell dimensions i.e., major and minor axes of cell and also the surface area and volume.

There has been a remarkable increase in the production and use of pesticides. In India the total consumption of technical grade pesticides was estimated at 58.540 tonns during the year 1976 (Krishna Murti et al. 1982). Since 1970, use of organochlorine pesticides (DDT, γ-HCH and dieldrin etc.) has decreased because of their major disadvantages: persistence in nature, build up in fatty tissues and broad spectrum of activity (i.e., their effect is not limited to target organisms but also extends to non-target organisms due to the ubiquitous nature of many biological processes). The organochlorine pesticides have progressively been replaced by organophosphorus compounds, which are more specific, more effective and less persistent in nature. In 1970, there were 140 commercially produced organophosphorus pesticides; of these phosphamidon is one of the systemic insecticides which is rapidly absorbed by the plants; it has little contact action and is widely used as a crop protectant in agriculture.

Insecticides are common contaminants of microbial environment. The protozoans are major component of aquatic microfauna and so form an important group of non-target organisms in the aquatic ecosystem. Ciliates form the base of many aquatic food chains. They are reported to accumulate insecticides to a concentration much higher than present in their immediate surroundigs (Lal 1982). Ciliates due to their rapid growth rate, ease of handling and genetic stability, have been used as toxicological tools in studies related to drug action mechanisms (Hunter 1964, Shivaji et al. 1975, Geike and Parashar 1976).

Tetrahymena pyriformis is common in ponds, fresh water lakes and slow moving streams, it may interact with the insecticide residue. Phosphamidon may also affect the general Protozoan community which is essential for sustaining the ecological balance. This paper deals with interaction of Phosphamidon with the Ciliate Protozoan *T. pyriformis*.

Material and Methods

T. pyriformis (Syngen I) was obtained from Dr J. G. Jones, Department of Biochemistry, University of Hull, Hull U. K. wayback in 1970. The axenic, asynchronous culture was maintained in autoclaved 1% proteose peptone medium (Difco), supplemented with 0.3% yeast extract and 0.5% Sodium Chloride at temperature 26 ± 1°C in a BOD incubator. The stock culture was subcultured once in a week by transferring few cells from a week old culture to 15 ml glass tube containing 5 ml of fresh sterile medium.

Ninety nine per cent purified samples of organophosphorus insecticide phosphamidon (2-chloro-2 diethyl carbamoyl-1-methyl vinyl dimethyl phosphate) have been obtained from Ciba-Geigy limited. The stock solutions of phosphamidon were prepared in a non-polar organic solvent acetone. Acetone concentration was kept 0.1 per cent as it was found to be non-toxic to normal cell population growth. The stocks of phosphamidon were stored in deep freezer at temperature minus 5°C.

The cell population growth of *Tetrahymena* in presence of phosphamidon was assessed continuously for five days at an interval of 24 h. The experimental culture was set up by transferring 4 loops (diameter 0.5 cm) full of 72 h old culture to 2 ml autoclaved medium in 15 ml centrifuge tubes. They were allowed to grow for 48 h and then transferred to 48 ml sterile medium in 100 ml conical flasks and allowed to grow for 24 hours. This is done to achieve log phase culture. Different concentration of phosphamidon were added separately from freshly prepared stock solutions to above mentioned cultures. The concentration of phosphamidon used ranged from 0.5 to 500 ppm. Simultaneously, two types of controls were run: (1) normal culture without phosphamidon and acetone and (2) culture containing 0.1 per cent acetone.

The number of cells per millilitre medium in each flask at the end of 24 h was determined by fixing 1 ml of the culture with an equal volume of 10% buffered neutral formalin and counting the cells on WBC counting surfaces of haemocytometer (Injeyan and Meerovitch 1974). Six to eight replicates were

kept for every concentration of phosphamidon used and out of these, two replicates were taken at one time and each of the replicates were counted thrice. Therefore, the cell counts used for calculations represent an average of six counts each. Experiments were repeated thrice.

For assessing reversibility of effects of phosphamidon on growth of *Tetrahymena*, recovery experiments were performed. The ciliates were exposed to sublethal concentrations i.e., 50 and 100 ppm of phosphamidon for 3 days and then after a thorough wash with Chalkley's isotonic salt solution consisting of w/v 0.1% NaCl, 0.0004% KCl, 0.0006% $CaCl_2$ (Randall and Jackson 1958) transferred to 50 ml toxicant free Chalkley's solution fortified with 0.3 percent yeast extract. The cell counts were taken after 1/2, 3, 6, 24, 36 and 48 h.

The effect of 50 and 100 ppm concentrations of phosphamidon on the cell dimensions i.e., major and minor axes, surface area and volume was determined using a calibrated oculometer. For average dimensions 25-30 formalin fixed cells were randomly selected and measured. Based on above measurements cell surface area and volume was computed using a formula for a prolate spheroid (Rohatgi and Krawiec 1973). Cell dimensions of fixed cells were comparable to live specimens.

Cell population growth study data was analysed statistically with least significant difference (LSD) at $P = 0.05$ for priori comparison of means among treatments (Sokal and Rohlf 1969).

Results

The pattern of cell population growth (Table 1) has been similar to that described by Cameron (1973). Since for this experiment log phase culture was used no lag phase was observed throughout the experiment. In *Tetrahymena* log phase lasts for four days followed by a stationary phase, the cells divide at a slower rate due to exhausted culture conditions. To avoid effects of depleted culture conditions on *T. pyriformis*, all experiments were run only for five days.

The graded concentrations of phosphamidon used was found to affect normal growth pattern of *T. pyriformis* (Fig. 1). Phosphamidon at 0.5 ppm did not have any significant effect on the cell number. At 1.0 and 2.5 ppm

Table 1

Normal growth pattern of *T. pyriformis* (Syngen I)

Number of days	Cell number $\times 10^4$/ml	
	normal control	acetone control
0	34.50	35.25
1	43.50	44.00
2	44.50	45.00
3	45.50	46.25
4	40.25	40.50
5	35.25	35.25

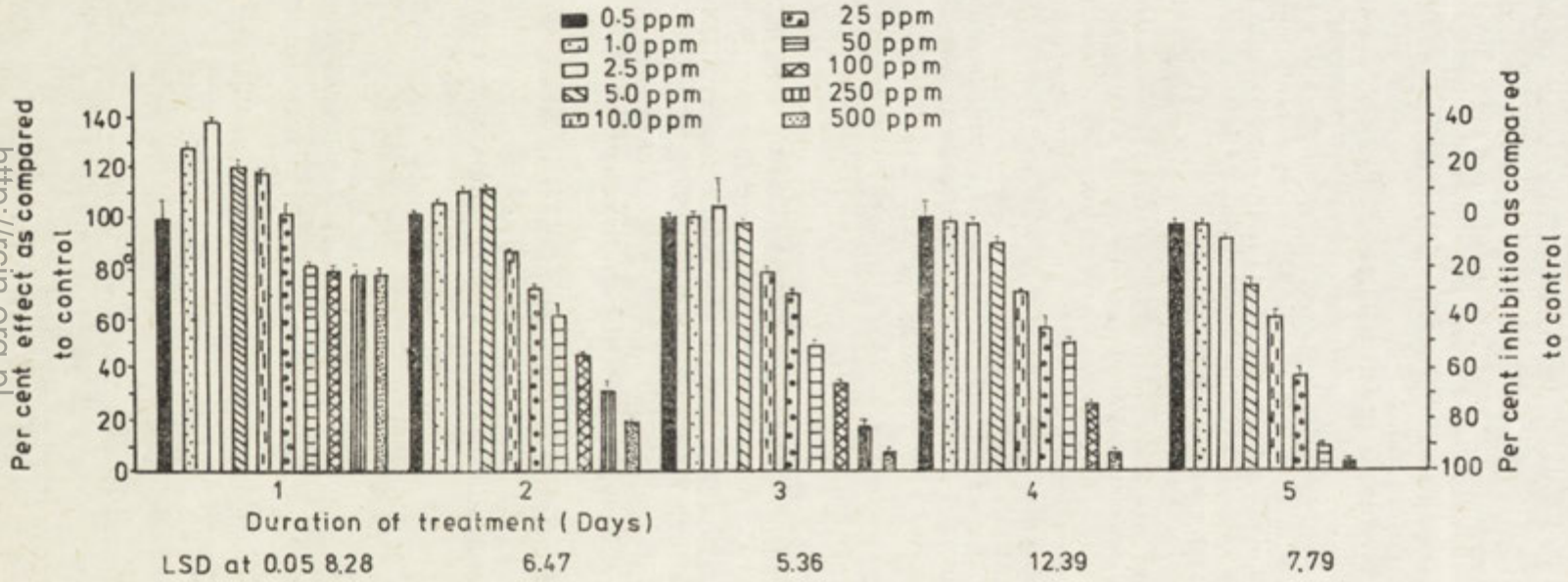

Fig. 1. Effect of phosphamidon on growth of *T. pyriformis*

an increase in cell count on the first and second day of treatment as compared to control and then a slight decrease on the fourth and fifth days was observed. Treatment with 5.10 and 25 ppm reduced cell count, the percent inhibition on fifth day as compared to acetone control was 27.40 and 63, respectively. At higher concentrations phosphamidon (50 and 100 ppm) resulted in reduction of cell number by 19 and 20 percent, respectively, on the first day and 90 and 97 percent, respectively, on the fifth day of treatment. Phosphamidon concentration higher than 250 ppm was lethal to *T. pyriformis*, as all cells died within four days of treatment. Percent inhibition in 500 ppm treated cultures from first to fourth day was 23, 81, 93 and 100, respectively.

A lot of cell lysis was observed in cultures treated with 10 and 50 ppm concentration, cytolysis was preceded by vacuolization and change in body contour from pyriform to spherical form. In 100-500 ppm treated cultures lot of dead cells in different states of disintegration were observed.

The major and minor axes and thus cell dimensions was found to be affected by phosphamidon (Fig. 2). In control major and minor axes of

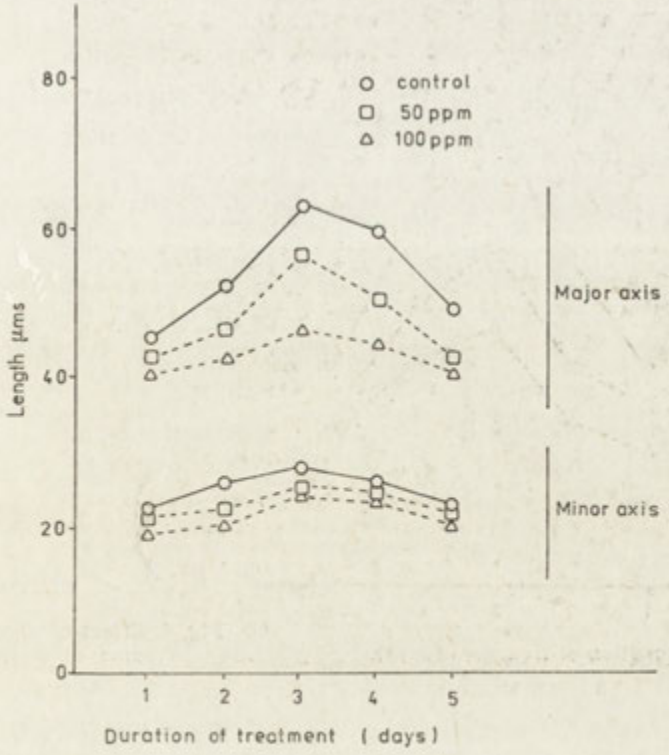

Fig. 2. Effect of phosphamidon on major and minor axes of *T. pyriformis*

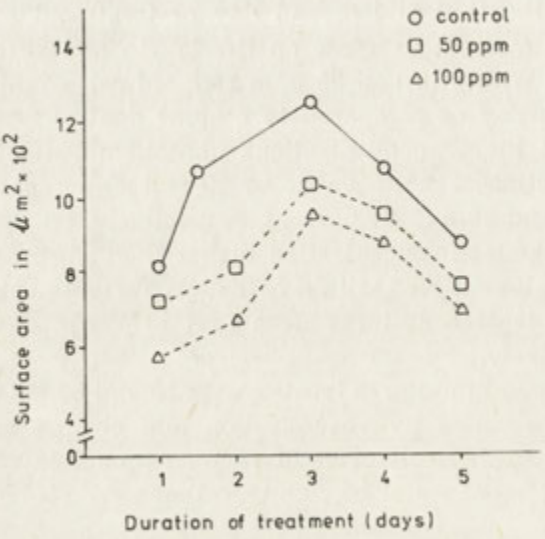

Fig. 3. Effect of phosphamidon on surface area of *T. pyriformis*

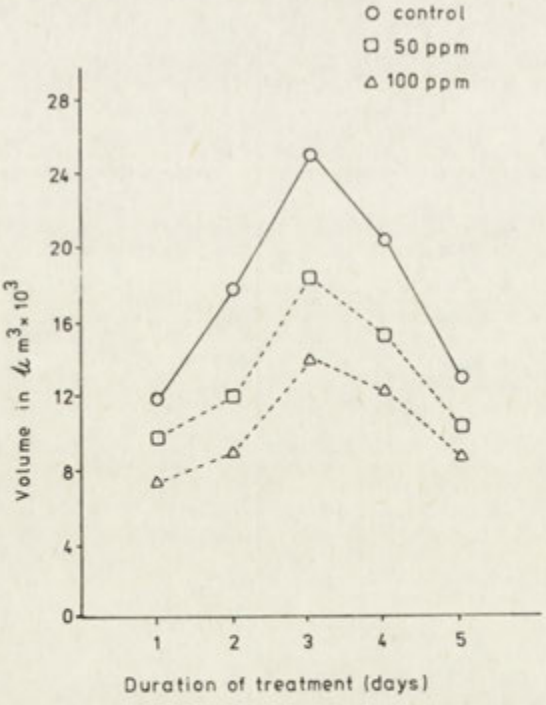

Fig. 4. Effect of phosphamidon on volume of *T. pyriformis*

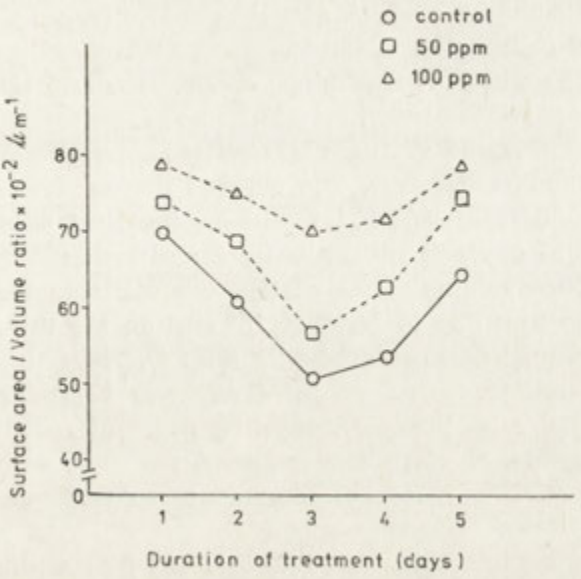

Fig. 5. Effect of phosphamidon on surface area/volume ratio of *T. pyriformis*

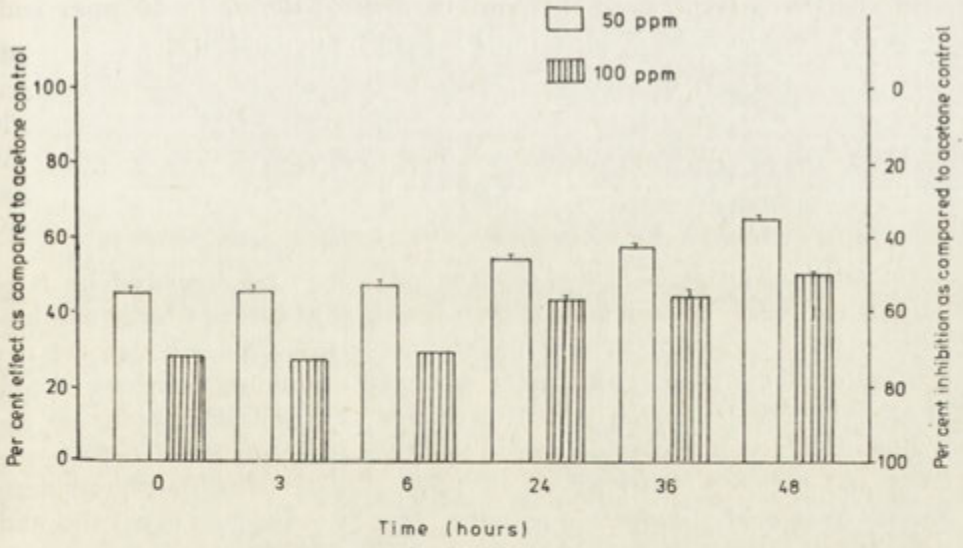

Fig. 6. Recovery of *T. pyriformis* after treated with phosphamidon for 3-days

T. pyriformis increased by 20% and 15% respectively in 72 h. The sublethal doses of phosphamidon (50 and 100 ppm) caused reduction in major axis by 11% and 27% and minor axis by 9% and 13%, respectively by 72 h as compared to control.

The surface area and volume also changed corresponding to the changes in major and minor axes. The average surface area in the acetone control increased from 8.04×10^2 μm^2 on the first day to 12.35×10^2 μm^2 on the third day and subsequently showed a decline (Fig. 3). Similarly, the average volume in the acetone control increased from 11.62×10^3 μm^3 on the first day to 24.76×10^3 μm^3 on the third day and there after the volume declined gradually (Fig. 4). The reduction in surface area as compared to control on third day was 17 and 20% at 50 and 100 ppm, respectively. The corresponding per cent reduction in volume ranged from 24-44. Surface area exposed per unit volume of treated cells was 12 and 38% more as compared to control cells (Fig. 5) in 50 and 100 ppm treated cultures, respectively.

T. pyriformis when transferred to toxicant free medium after treatment, started recovering within 6 h from the effect, i.e., they assumed normal shape, size and locomotion. Culture entered log phase of growth at the end of 36 h (Fig. 6) initially the increase in cell number was gradual and then it was comparable to the control. The cell count on recovery increased from 18.33×10^4/ml to 31.00×10^4/ml in 50 ppm and 11.13×10^4/ml to 23.86×10^4/ml in 100 ppm treated cultures.

Discussion

Insecticidal properties of phosphamidon were first described by Bachmann and Meierhan's (1956). It is highly toxic to birds and honey bees and is one of the organophosphorus insecticides which are reported to be the general inhibitors of serine containing enzymes (Bell et al. 1970) Phosphamidon though functions as a acetylcholinesterase inhibitor in addition it also decreases the glycogen reserves of hepatopancreas and muscles (Omkar et al. 1986), inhibits alkaline phosphatase and elevates acid phosphatase activity (Sastry and Malik 1979) and decreases the total amino acid content (Omkar 1983). Thus phosphamidon exposure causes widespread physiological disturbances which ultimately causes lethal effects.

Exposure of *T. pyriformis* to graded concentration of phosphamidon inhibited cell population growth. Other insecticides such as DDT, γ-HCH,

etc. are known to retard growth of Protozoans, e.g., *Euplotes* (Personne and Uyttersprot 1975), *Crithidia fasciculata* (French and Roberts 1976), *Stylonychia notophora* (Lal and Saxena 1980), *Tetrahymena pyriformis* Lal and Saxena 1979, Mathur et al. 1984, Saini and Saxena 1986), *Paramecium aurelia* (Tandon et al. 1987).

The exact mechanism of interaction of organophosphorus insecticides with *T. pyriformis* is not known. However, there are reports regarding site of action of other insecticides. DDT is known to bind with plasma membrane and affects its lipid bilayer (Hick and Corner 1973, French 1976), ratio of polar head groups and composition of fatty acids (Rosas et al. 1980), uptake of amino acids (Czeczuga and Gierosimow 1977) and synthesis of nucleic acide (French 1976). DDT and γ-HCH interact with lysosomal membrane resulting in release of active hydrolytic enzymes and ultimately cell lysis (Carviec 1977). Work of Prevot and Soyer (1985) reveals that acetylcholinesterase enzyme is absent in marine dinoflagellate *Prorocentrum micans* Ehrbg. and so it could not be possible target site of parathion action. For phosphamidon Sastri and Malik (1979) have shown an increase in acid phosphatase activity in cells of digestive system of a fresh water fish *Channa punctatus*.

Both microbs and higher organisms have been shown to recover from the toxic effects of the insecticides once transferred to the medium free from it. *T. pyriformis* after treatment with sublethal concentrations of phosphamidon recovers from the shocks of toxicant within six hours of transfer. The mechanism of recovery is not known, however, it is established that *T. pyriformis* is capable of metabolizing and eliminating DDT from the surroundings (Agarwal et al. 1981).

Cell shape of *T. pyriformis* changes from prolate spheroid to spherical. Major and minor axes were less as compared to the control cells. Consequently, surface area and volume were also less. Surface area to volume were also less. Surface area to volume ratio was more than control cells on third day of treatment. Rohatagi and Krawiec (1973) suggested such changes occur as a natural response of the cell to the adverse environmental conditions which may arise from the catabolism of the polymer reserves.

In the treated cultures we observed more spherical cells than prolate spheroids. The cell count was also very low. Watanabe (1971) has reported formation of spherical cells in *T. pyriformis* on preventing cell division by amino acid starvation. It is quite probable that phosphamidon blocks one or more vital reaction concerned with cell division which results in a very low cell count.

REFERENCES

Agarwal H. C., Saxena D. M. and Rup Lal. 1981: Accumulation and metabolism of DDT and its metabolites by *Tetrahymena*. Water. Air Soil Pollution., 18, 441-447.

Bachmann F. and Meierhans J. 1956. Bull. Cent. Int. Antiparasit., 18.

Bell G. H., Davison J. N. and Smith D. E. 1970: Text Book of Physiology and Biochemistry. 8th ed. Tinling, London.

Cameron I. L. 1973: Growth characteristic of *Tetrahymena*. In: Biology of *Tetrahymena* (Ed. A. M. Elliott), Dowden, Hutchinson and Ross. Inc. Stroudsburg, Pennsylvania, 199-226.

Carviec O. 1977: The effect of gamma hexachlorocyclohexane on rat liver lysosomes. Biochem. Pharmacol., 15, 1377-1381.

Czeczuga B. and Gierasimow M. 1977: Wpływ DDT na zawartość aminokwasów w hodowlach zielenic jednokomórkowych. Bromatol. Chem. Toksykol., 10, 331-337.

French J. E. 1976: The effect of the chlorinated polycyclic hydrocarbons p-p DDT and polychlorinated biphenyles (PCB's) on the flagellated protozoan *Crithida fasciculata*. Diss. Abstr. Int., B 13, 37, 96-97.

French J. E. and Roberts J. F. 1976: Effect of DDT and polychlorinated biphenyls on cellular metabolism and ultrastructure of *Crithidia fasciculata*, a flagellated protozoan. Natl. Techn. Inf. AD-AO 33507, 26.

Geike F. and Parashar C. D. 1976: Effect of hexachlorobenzene (HCB) on growth of *Tetrahymena pyriformis*. Bull. Environ. Contam. Toxicol., 16, 347.

Hick C. F. and Corner T. R. 1973: Location and consequences of 1,1,1 trichloro 2,2', bis (p. chlorophenyl) ethane uptake by *Bacillus magaterium*. Appl. Microbiol., 25, 381-387.

Hunter S. H. 1964: Protozoa as toxicological tools. J. Protozool., 11, 1-6.

Injeyan H. S. and Meerovitch E. 1974: The effect of juvenile hormone on the growth of some protozoans in vitro I. *Crithidia* sp. J. Protozool., 21, 738-742.

Krishna Murti C. R., Diksit T. S. S. and Surya Prakash. 1982: Status of research on biodegradable pesticides in India. In: Proceedings of Indo U. S. workshop on biodegradable pesticides. (Eds. B. S. Attri, N. L. Ramanathan and C. R. Krishna Murti), Department of Environment, Government of India, New Delhi, 1-70.

Lal R. 1982. Accumulation, metabolism and effect of organophosphorus insecticides on microorganisms. Adv. Appl. Microbiol., 28, 149-200.

Lal R. and Saxena D. M. 1979: Effect of DDT on growth of *Tetrahymena pyriformis*. Arch. Protistenk., 122, 382-386.

Lal R. and Saxena D. M. 1980: Effect of DDT on cell population growth cell division and DNA synthesis in *Stylonychia notophora* (stokes). Arch. Environ. Contam. Toxicol., 9, 163-170.

Mathur R., Saxena D. M. and Agarwal H. C. 1984: Growth of a ciliate protozoan, *Tetrahymena pyriformis* in the presence of different isomers of hexachloro-cyclohexane (HCH). Acta Protozool., 23, 165-174.

Omkar 1983: Effects of some insecticides on certain systems of freshwater prawn *Macrobrachium lamarrei* (H. Milne Edwards), Ph. D. thesis, Gorakhpur University, Gorakhpur, India.

Omkar, Upadhyay V. B. and Shukla G. S. 1986: Impact of phosphamidon on the carbohydrate metabolism of fresh water prawn *Macrobrachium lamarrei*. Environ Research, 41, 591-597.

Personne G. and Uyttersprot G. 1975: The influence of organic and inorganic pollutants on the rate of reproduction of marine hypotrichous ciliate, *Euplotes vannus*. Res. Oceanogr. Med., 37, 125-151.

Prevot P. and Soyer M. D. 1985: Action of organophosphorus insecticide parathion on the free living marine dinoflagellate *Prorocentrum micans* Ehrbg. J. Protozool., 32, 626-633.

Randall J. F. and Jackson S. 1958. Fine structure in *Stentor polymorphus*. J. Biophys. Biochem. Cytol., 4, 807.

Rohtagi H. and Krawiec S. 1973: Some effects of chloramphenicol and ethidium bromide in Tetrahymena pyriformis. J. Protozool., 20, 425-430.

Rosas S. B., Secco M. D. C. and Ghittoni N. E. 1980: Effects of pesticides on the fatty acids and phospholipids composition of *Escherichia coli*. Appl. Environ. Microbiol., 40, 231-234.

Saini A. and Saxena D. M. 1986: Effect of organophosphorus insecticides on the growth of *Tetrahymena pyriformis*. Arch. Protistenk., 131, 143-152.

Sastry K. V. and Malik P. V. 1979: Studies on the effect of dimecron on the digestive system of a fresh water fish, *Channa punctatus*. Arch. Environ. Contam. Toxicol., 8, 397-407.

Shivaji S., Saxena D. M. and Pillai M. K. K. 1975: Mode of action of alkylating agents using ciliate protozoan as a model system. I. Effect of metepa on cell division and DNA synthesis in *Stylonychia notophora* (*Ciliata: Hypotrichida*). Arch. Protistenk., 117, 303.

Sokal R. R., and Rohlf F. J. 1969: In: The principles and practice of statistics in biological research (Eds. R. Emerson, D. Kennedy and R. B. Pork).

Tandon R. S., Lal R. and Narayana Rao V. V. S. 1987. Effects of malathion and endosulfan on the growth of *Paramecium aurelia*. Acta Protozool., 26, 325-328.

Watanabe Y. 1971: Mechanism of synchrony induction. I. Some features of synchronous rounding of *Tetrahymena pyriformis*. Cell Res., 68, 431-436.

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Electron Microscopy of Jejunum of Rats Infected Experimentally with *Giardia muris*

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Synopsis. This electron microscopy study of small intestine complemented the previous light microscopy study conducted in the rat infected experimentally with *Giardia muris*. Most epithelial cells of the jejunal mucosa showed normal appearance and the changes observed in electron microscopy corresponded with the light microscopy findings. They were slight and focal, however, the peak of their intensity fell on a more distant period of the infection. We found all the changes in fine structure which had been described in human jejunal biopsies, and also other hitherto not observed (swelling of mitochondria, numerous undifferentiated enterocytes with cytoplasm free of organelles, and proliferation of mucosal mast cells which was accompanied by a small cellular infiltration). The trophozoites of *Giardia* were not seen within the intestinal mucosa. The alterations of microvilli in sections of jejunum where numerous *Giardia* were found testify to a direct effect of the parasite. It seems that the changes in fine structures of jejunal enterocytes resulted as a consequence of a prolonged course of giardiasis in rats, characterized by a stable although not very high intensity of infection Our electron microscopic study indicates that the rat may be a useful model of giardiasis characterized by a long-termed chronic infection, reflecting in mild although composed changes of fine structure of the jejunal mucosa.

In a study conducted in the rat model of giardiasis we found that the infection is characterized by a long-term chronic course, without negative stools and spontaneous elimination of the parasite (Majewska

and Kasprzak 1983, Majewska 1985). Minor morphological changes were observed in the jejunum, however, of particular interest was the proliferation of mucosal mast cells (Kasprzak, Majewska, Gustowska and Ruitenberg in preparation). Because it was concluded by the authors that experimental rat might serve as a model for long-standing *Giardia* infection, the electron microscopy study of small intestine was also performed.

Material and Methods

Parasite. *G. muris* cysts were obtained from fresh feces of cyst-passing rats infected experimentally with a strain originally isolated from a spontaneously infected Wistar rat and subsequently passed repeatedly through Wistar rats (Majewska and Kasprzak 1983). Cyst inoculum was prepared by a modified procedure of Meyer (1979) and described elsewhere (Majewska and Kasprzak 1983). The final pellet was resuspended in water and cysts were counted in a hematocytometer chamber. The volume of the inoculum was 0.1 ml and the number of cysts was about 10000. However, the number of viable cysts was about 1000 (10%) when calculated with the use the excystation procedure (Bingham and Meyer 1979) and about 9500 (95%) when determined by the eosin-exclusion method (Kasprzak and Majewska 1983).

Animals. Three-week-old weanling outbred Wistar rats of either sex (Institute of Animal Breeding, SGGW-Academy of Agriculture Warsaw, Brwinów), weighing 25-30 g, were used. The animals, derived from a stock free of any natural parasitic infections and *Giardia*-free for six generations, were maintained in parasite-free conditions (sterilized food, water, cages, and bedding), and fed on standard pellet diet (LSM) and water ad lib. The non-infected and infected rats were housed in separate boxes.

Experimental design. The rats were inoculated intragastrically by intubation with 10000 *Giardia* cysts each. The infection was confirmed by microscopical examination of feces (sucrose flotation procedure by Bingham and Meyer 1979); the parasitological examinations were performed daily with fresh feces and the number of cysts was estimated semiquantiatively as + (few cysts in whole preparation), ++ and +++ (1 — 5 cysts and over 5 cysts per microscopic field respectively). As a control served non-infected animals. For ultrastructural studies the rats were divided in five groups of two infected and two non-infected animals each, and killed on day 7, 14, 28, 42 and 56 post infection (p. i.).

Electron microscopy. The rats were killed by overdosing with ether, segments of jejunum were removed, dissected longitudinally, and samples were fixed in 3.6% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 12-20 hours. After washing in 0.1 M cacodylate buffer (pH 7.4) for 24 hours the samples were postfixed with 1% (v/v) osmium tetroxide in phosphate buffer (pH 7.4), dehydrated stepwise in ethanol-acetone, embedded in Epon 812 resin, and polymerised at 60°C for 2 weeks. Thin sections were cut on an Reichert ultramicrotome, and examined with a JEM7A transmission electron microscope at 80 kV.

Results

Parasitological examinations

Daily microscopic examination of feces showed that the infection was of long duration; all animals were infected during the whole period of experiment, and the pattern of cyst excretion showed little variability. *Giardia* cysts were found in the feces begining from day 4 p. i. The number of cysts was not high, but rose by the second week and remained on that level ("+" in 65 — 85% of samples) to the end of experiment.

Transmission electron microscopy

Group 1: 7 days p.i. Most epithelial cells of the jejunal mucosa showed normal appearance, and, on the whole, the villi were normal. There were little changes of intestinal epithelium manifested by swelling of mitochondria (Pl. I 1) and rupture of cell membrane (Pl. I 1). Numerous enterocytes with cytoplasm free of organelles were found (Pl. I 2). The microvilli of some enterocytes were shortened and fewer, particularly in these sections of intestine where numerous *Giardia* were found (Pl. II 3). The luminal surface of intact microvilli of enterocytes was sometimes deformed by the edges of the parasite's adhesive disc (Pl. III 4). The number of figures of mitotic division of the nuclei of enterocytes was increased. There was little infiltration of intercellular spaces with epithelial cells composed of eosinophils, lymphocytes, and single macrophages, and also of lamina propria with eosinophils, lymphocytes, plasma cells, smooth muscle cells, and fibroblasts. Also enterochromaffin cells with dense granules were found more frequently (Pl. IV 5). The trophozoites of *Giardia* were not seen within the intestinal mucosa.

Group 2: 14 days p.i. The changes of intestinal epithelium resembled that on day 7 p. i., however, they were more dictinct and concerned greater number of cells. Between the damaged enterocytes, mastocytes and dense cells with homogenous cytoplasm — the identification of which was unfeasible — were present (Pl. IV 6). Some enterocytes produced evaginations directed to the opposite side of the intestinal lumen (Pl. IV 7).

Group 3: 28 days p.i. Similar changes were observed as in preceding period. The mast cells were found also in lamina propria (Pl. V 8).

Group 4: 42 days p.i. The changes of jejunal mucosa persisted, end even increased. The cisternae and labyrinthine network of endoplasmic reticulum were widened (Pl. V 9), and the number of enterocytes with cytoplasm free of organelles increased. Lamina propria was slightly infiltrated mainly by plasma cells and smooth muscle cells. Some ente-

rocytes contained electron-dense granules lying within the endoplasmic reticulum (Pl. V 10).

Group 5: 56 days p.i. Small numbers of damaged enterocytes were found. In the part of intestinal epithelium adjacent to lamina propria the intercellular spaces were widened and filled with electron-dense material. In the vicinity of blood capillaries fibrin deposits were observed, which evidenced the intravascular coagulation (Pl. V 11).

Discussion

In a study conducted with originally *Giardia*-free rats infected experimentally with *Giardia muris* we observed a long duration of the infection, without spontaneous elimination of the parasite and without decrease in cyst excretion (Majewska and Kasprzak 1983, Majewska 1985). Light microscopy of jejunal mucosa showed only slight and focal changes, however, a significant increase in the number of mucosal mast cells was observed (Kasprzak, Majewska, Gustowska and Ruitenberg, in preparation). The changes observed in electron microscopy coresponded to the light microscopy findings; they were slight and focal, however, the peak of their intensity fell on subsequent period of the infection (42nd day p. i.).

The electron microscopy in biopsies of human infected with *G. intestinalis* usually showed no changes (Klima et al. 1977, Gillon and Ferguson 1984) or slight changes of jejunal mucosa involving, as a rule, single abnormalities of cells (Kasprzak et al. 1987): microvilli branching and gaps between their bases (Barbieri et al. 1970), shorteness and distortion of brush border (Takano and Yardley 1965, Hoskins et al. 1967, Barbieri et al. 1970, Tubbs and Hawk, 1976), thickening of the mucoid coat ("fuzzy coat") of the microvilli (Barbieri et al. 1970, Balázas and Szatlóczky 1978), interruption and undulation of lateral membranes of mucosal cells (Morecki and Parker 1967), increased number of cytoplasm dense bodies, probably lysosomes (Barbieri et al. 1970), widening of cisternae of endoplasmic reticulum (Morecki and Parker 1967, Kocięcka et al. 1984, 1985), widening of intercellular spaces (Kocięcka et al. 1984), and little infiltration of intercellular spaces and lamina propria with inflamatory cells (Barbieri et al. 1970, Kocięcka et al. 1984, 1985).

In our experiments on rat giardiasis the alterations of jejunal mucosa cells were mild and focal indeed, however, rather composed; we found all the changes in fine structures which have been described in human jejunal biopsies, and also other hitherto not observed such as swelling

of mitochondria and numerous undifferentiated enterocytes with cytoplasm free of organelles. It seems that the changes in fine structures of jejunal enterocytes observed in this study resulted as a consequence of a prolonged course of giardiasis in rats, characterized by a stable although not very high intensity of infection. The changes of jejunal mucose persisted for a longer period (until the and of the observation) when compared with the light microscope observations (Kasprzak, Majewska, Gustowska and Ruitenberg, in preparation).

Ferguson and co-workers (1980) concluded that the chronic giardiasis resulted in doubling of the crypt cell production rate and in rapid transit of the udifferentiated enterocytes up the villi. Also other authors found an increased epithelial mitotic index (Yardley et al. 1964). The numerous enterocytes with cytoplasm free of organelles and increased number of figures of mitotic division in this study confirmed the enhancement of mitotic activity of intestinal epithelial cells.

The widening of cisternae and canaliculi of endoplasmic reticulum we found, similarly to Morecki and Parker (1967), at the late course of giardiasis may be related to increased mucoid coat of the epithelial cells as a defensive reaction to avoid the parasite's invasion (Barbieri et al. 1970, Tandon et al. 1974).

An interesting observation was the proliferation of mucosal mast cells, which was accompanied by a small cellular infiltration, as observed also in our histopathological study (Kasprzak, Majewska, Gustowska, Ruitenberg, in preparation). Anand and others (1985) supposed that it appears unlikely that the damage to the small intestinal epithelial cells could be mediated by immunological reactions. However, markedly raised intraepithelial lymphocyte counts were found exceptionally by some authors in *Giardia* infection with malabsorbtion (Wright and Tomkins 1977, Ferguson et al. 1976, Ganguly et al. 1985).

The alterations of microvilli in sections of jejunum where numerous *Giardia* were found testify to a direct effect of the parasite, which is in accordance with the observations of other authors (Morecki and Parker 1967, Barbieri et al. 1970, Holberton et al. 1973, Anand et al. 1985). The deformation of the surface of intact microvilli by the parasite's adhesive disc, which we have observed, confirms this mechanism responsible for the mucosal abnormalities. Some authors found circular impressions at the site of the attachment of the parasite's disc (Erlandsen and Chase 1974, Owen et al. 1979).

We have not found *Giardia* penetrating between epithelial cells or into deeper tissues, although such tissue penetration has been reported by other authors (Brandborg et al. 1967, Morecki and Parker

1967, Owen et al. 1979, 1981, Fleck et al. 1985). Taking into consideration the large number of trophozoits living within the intestinal lumen, the invasion of the walls seems to be an occasional, infrequent occurrence.

Our electron microscopic study indicate that the rat may be a useful model of giardiasis, characterized by a long-termed chronic infection, without negative stools and spontaneous elimination of the parasite, reflecting in mild, however, composed changes of fine structure of the jejunal mucosa.

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REFERENCES

Anand B. S., Chaudhary R., Jyothi A., Yadev R. S. and Baveja U. K. 1985: Experimental examination of the direct damaging effects of *Giardia lamblia* on intestinal mucosal scrapings of mice. Trans. Roy. Soc. Trop. Med. Hyg., 79, 613-617.

Balázas M. and Szatlóczky E. 1978: Electron microscopic examination of the mucosa of the small intestine in infection due to *Giardia lamblia*. Pathol. Res. Practice, 163, 251-260.

Barbieri D., DeBrito T., Hoshino S., Nascimento Fa O. B., Martins Campos J. V., Quarentei G. and Marcondes E. 1970: Giardiasis in childhood. Absorption tests and biochemistry, histochemistry, light, and electron microscopy of jejunal mucosa. Arch. Dis. Childhood, 45, 466-472.

Bingham A. K. and Meyer E. A. 1979: *Giardia* excystation can be induced in vitro in acidic solutions. Nature, 277, 301-302.

Brandborg L. L., Tankerley C. B., Gottlieb S., Barnacik M. and Sartor V. E. 1967: Histological demonstration of mucosal invasion of *Giardia lamblia* in man. Gastroenterology, 52, 143-150.

Erlandsen S. L. and Chase D. G. 1974: Morphological alternations in the microvillous border of villous epithelial cells produced by intestinal microorganisms. Am. J. Clin. Nutr., 27, 1277-1286.

Ferguson A., Gillon J. and Al Thamery D. 1980: Intestinal abnormalities in murine giardiasis. Trans. Roy. Soc. Trop. Med. Hyg., 74, 445-448.

Ferguson A., Mc Clure J. P. and Townley R. R. 1976: Intraepithelial lymphocyte counts in small intestinal biopsies from children with diarrhoea. Acta Pediatr. Scand., 65, 541-546.

Fleck S. L., Hames S. E. and Warhurst D. C. 1985: Detection of *Giardia* in human jejunum by the immunoperoxidase method. Specific and non-specific results. Trans. Roy. Soc. Trop. Med. Hyg., 79, 110-113.

Ganguly N. K., Mahajan R. C., Radhakrishna V. and Bhagwat A. G. 1985: Electron microscopic studies of jejunum of mice infected with *Giardia lamblia*. Indian J. Med. Res., 81, 102-110.

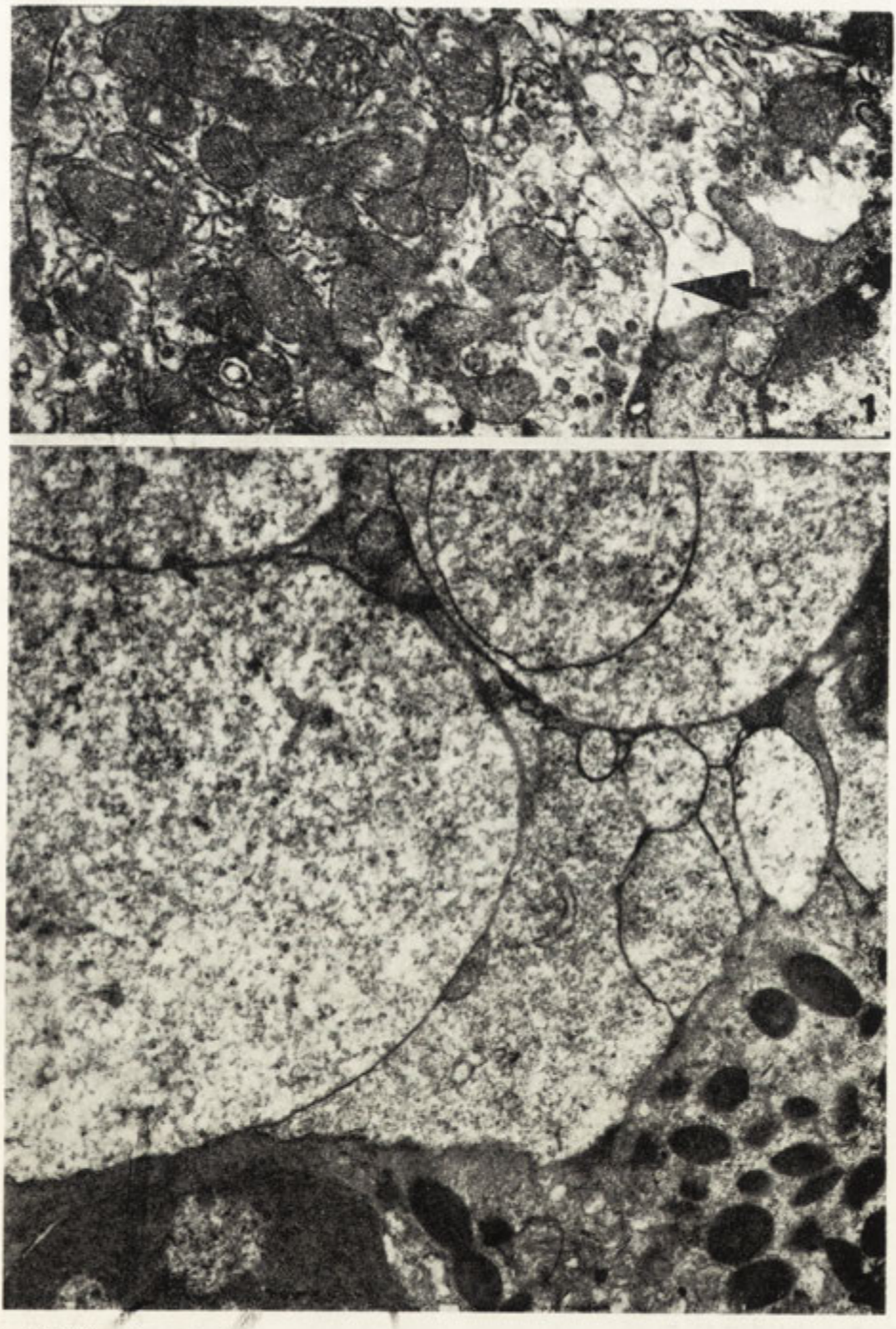
Gillon J. and Ferguson A. 1984: Changes in the small intestinal mucosa in giardiasis. In: Giardia and giardiasis — biology, pathogenesis, and epidemiology. (Eds. S. L. Erlandsen and E. A. Meyer), NY USA and London UK, Plenum Press, 163-183.

Holberton D. V. 1973: Fine structure of the ventral disk apparatus and the mechanism of attachment in the flagellate *Giardia muris*. J. Cell Sci., 13, 11-41.
Hoskins L. C., Winawer S. Y., Broitman S. A., Gottlieb L. S. and Zamcheck N. 1967: Clinical giardiasis and intestinal malabsorption. Gastroenterology, 53, 265-279.
Kasprzak W., Kocięcka W. and Majewska A. C. 1987: Patogeneza giardiazy. Pol. Tyg. Lek., 42, 1067-1070.
Kasprzak W. and Majewska A. C. 1983: Infectivity of *Giardia* sp. cysts in relation to eosin exclusion and excystation in vitro. Tropenmed. Parasit., 34, 70-72.
Klima M., Gyorkey P., Kyung-Whan Min and Gyorkey F. 1977: Electron microscopy in the diagnosis of giardiasis. Arch. Path. Lab. Med., 101, 133-135.
Kocięcka W., Błotna M., Gustowska L. and Knapowska M. 1985: Przewlekłe zakażenie *Giardia intestinalis*. Pol. Tyg. Lek., 40, 657-660.
Kocięcka W., Gustowska L., Gawroński M., Błotna M. and Knapowska M. 1984: Evaluation of jejunal mucosa biopsy in patients with giardiasis. Wiad. Parazytol., 30, 311-319.
Majewska A. C. 1985: *Giardia muris* u szczura jako model giardiazy. Wiad. Parazytol., 31, 27-49.
Majewska A. C. and Kasprzak W. 1983: The rat as an animal model of giardiasis. Acta Protozool., 22, 71-77.
Meyer E. A. 1979: Determination of *Giardia* cyst viability. In: Environmental Protection Technology, Research Report. US EPA, Cincinnati, Ohio.
Morecki R. and Parker J. G. 1967: Ultrastructural studies of the human *Giardia lamblia* and subjacent jejunal mucosa in a subject with steatorrhea. Gastroenetrology, 52, 151-164.
Owen R. L., Allen C. L. and Stevens D. P. 1981: Phagocytosis of *Giardia muris* by macrophages in Peyer's patch epithelium in mice. Inf. Immun., 33, 591-601.
Owen R. L., Nemanic P. C. and Stevens D. P. 1979: Ultrastructural observations on giardiasis in a murine model. I. Intestinal distribution, attachment, and relationship to the immune system of *Giardia muris*. Gastroenterology, 76, 757-769.
Takano J. and Yardley J. H. 1965: Jejunal lesions in patients with giardiasis and malabsorption: an electron microscopic study. Bull. Johns Hopkins Hosp., 116, 413-429.
Tandon B. N., Puri B. K., Gandhi P. C. and Tewari S. G. 1974: Mucosal surface injury of jejunal mucosa in patients with *Giardia* infection: an electron microscopic study. Indian J. Med. Res., 62, 1838-1842.
Tubbs R. R. and Hawk W. A. 1976: Surface ultrastructure of giardial infestation. Cleveland Clin. Quart., 43, 293-299.
Wright S. G. and Tomkins A. M. 1977: Quantification of the lymphocytic infiltrate in jejunal epithelium in giardiasis. Clin. exp. Immunol., 29, 408-412.
Yardley J. H., Takano J. and Hendrix T. R. 1964: Epithelial and other mucosal lesions of the jejunum in giardiasis. Jejunal biopsy studies. Bull. Johns Hopkins Hosp., 115, 389-406.

EXPLANATION OF PLATES I—V

Changes in fine structures in jejunum of rats infected experimentally with *Giardia muris*.

1: Swelling of mitochondria and rupture of cell membrane (arrow). × 15000
2: Enterocytes with cytoplasm free of organelles. × 15000
3: Enterocytes with shortened and fewer microvilli (in the upper part are numerous *Giardia*). × 16500
4: Enterocytes brush border deformed by the edges of adhesive disc of *Giardia*. × 8750
5: An enterochromaffin cell (with dense granules). × 15000
6: A dense cell with homogenous cytoplasm. × 11500
7: Enterocyte with evaginations directed to the opposite side of the intestinal lumen. × 12500
8: Mast cell lying in lamina propria. × 11500
9: Widening of cisternae and canaliculi of endoplasmic reticulum. × 8400
10: Enterocytes containing electron-dense granules lying within the endoplasmic reticulum. × 12500
11: Fibrin deposites in intercellular species (arrow). × 11500



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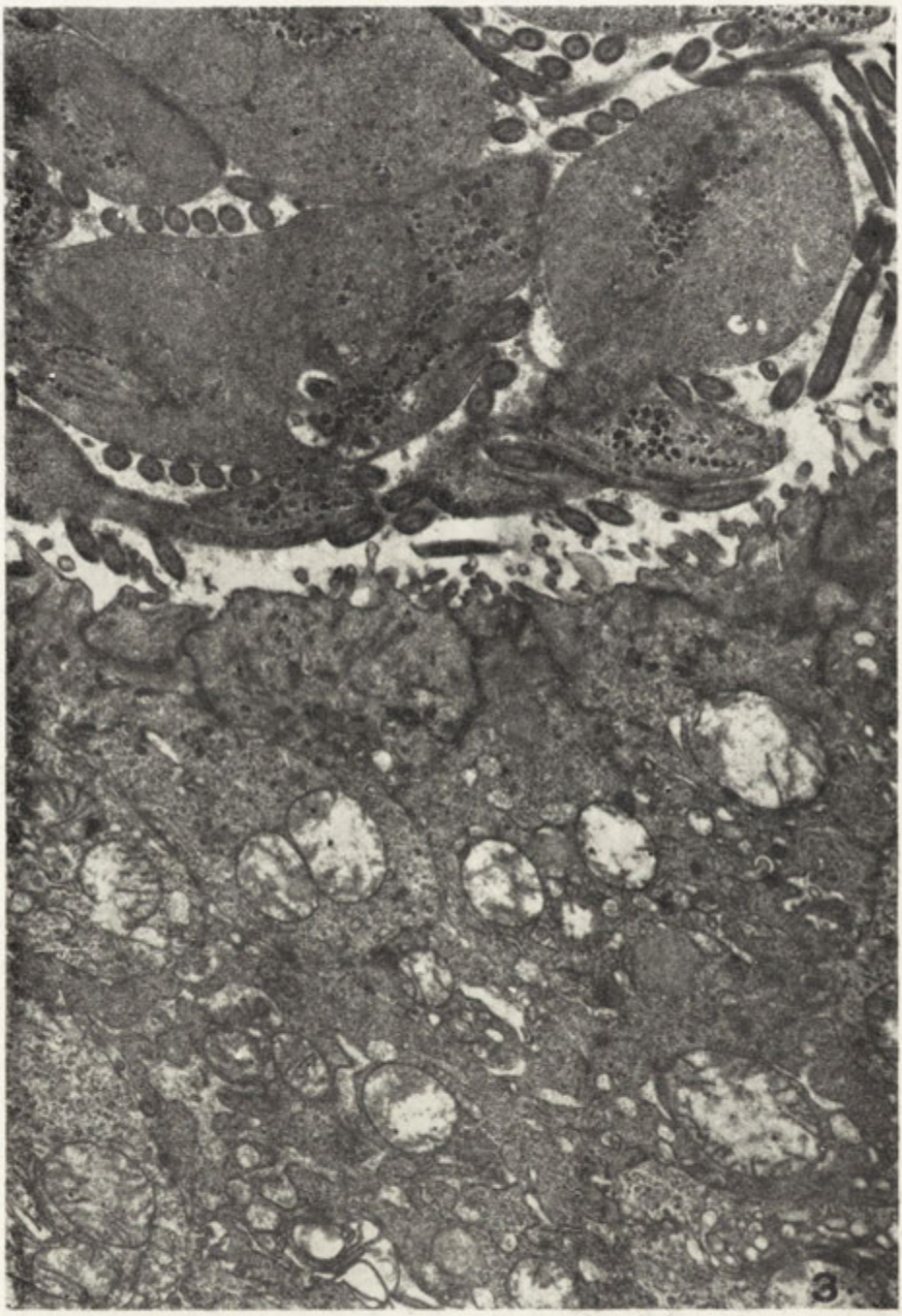

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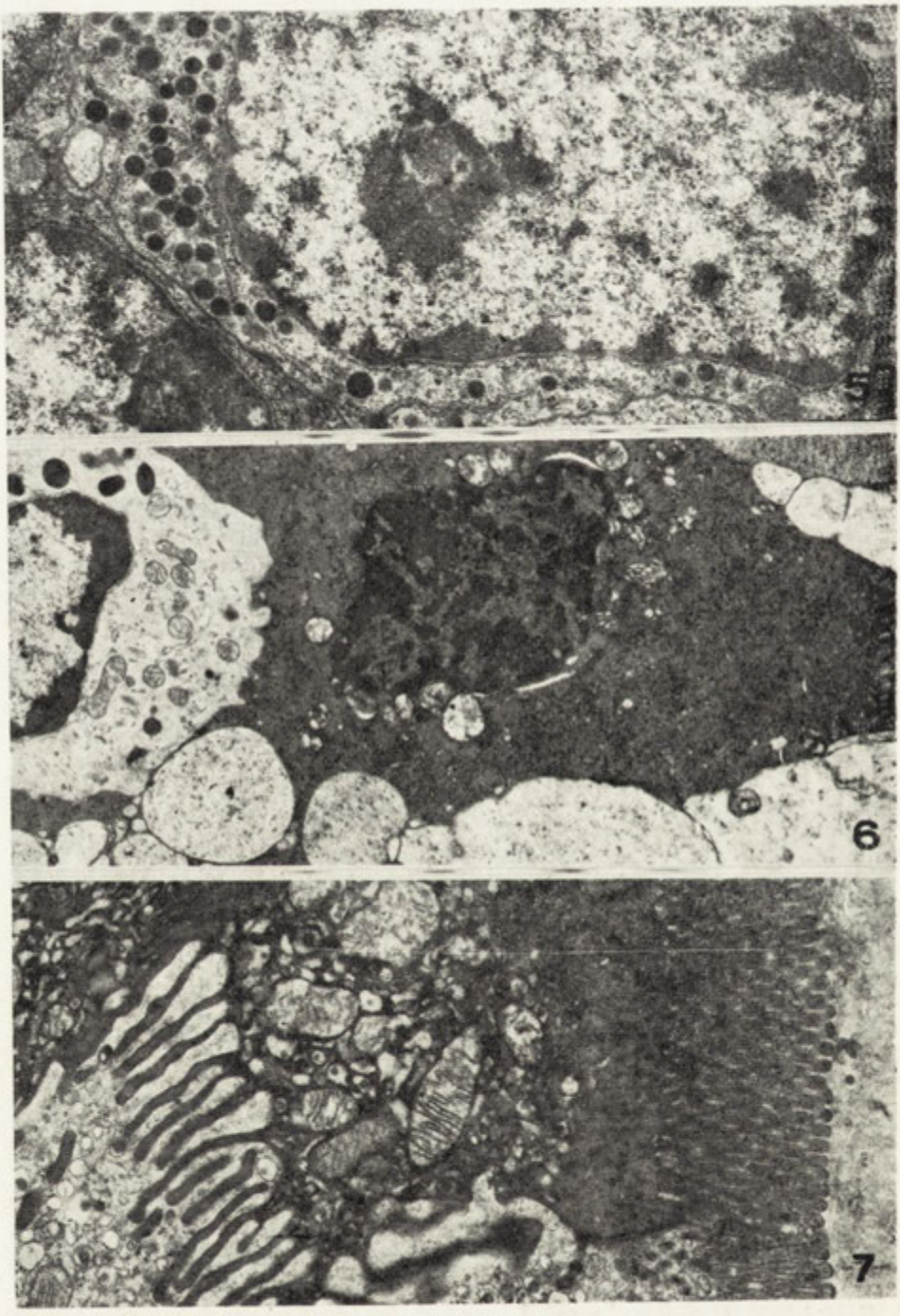

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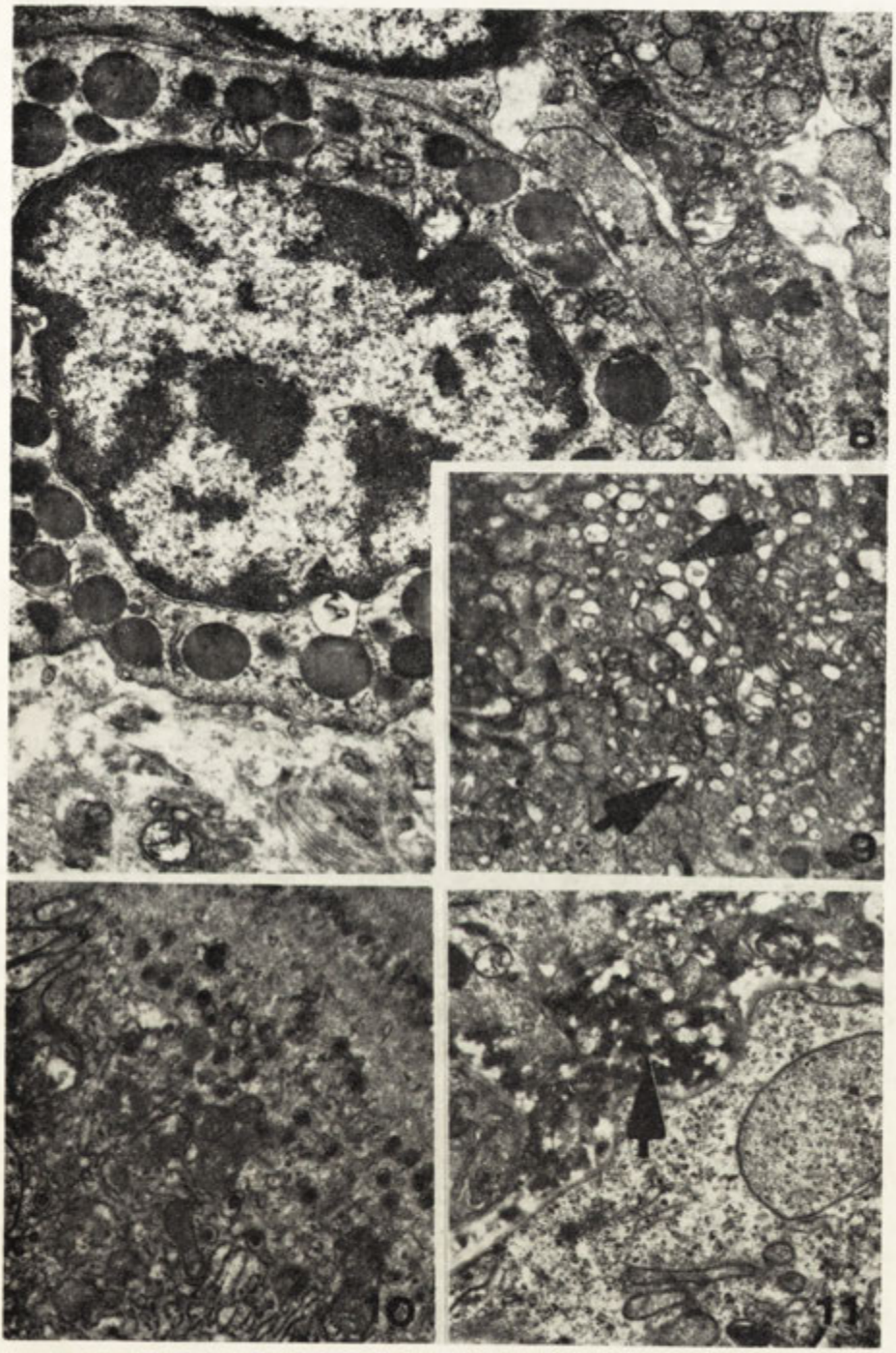

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Notes sur *Cyphoderia perlucidus* Beyens et Chardez, 1986 et sur le périphyton des plantes aquatiques

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Synopsis. Nous étudions le périphyton des plantes aquatiques considéré comme niche écologique particulière. Nous apportons quelques précisions sur la biologie de *Cyphoderia perlucidus* et des espèces qui l'accompagnent habituellement.

Cyphoderia perlucidus, observé pour la première fois parmi des Mousses très humides provenant de l'Ile Edgeøya (Arctique), a été revu dans plusieurs stations en Belgique, souvent isolé dans le sapropèle à la base des plantes aquatiques, mais principalement en population dans le dépôt sur les plantes immergées. Ce périphyton semble être son milieu habituel. Penard (1902), signale ce milieu comme étant apprécié par de nombreuses espèces de Thecamoebiens.

L'étude de ce biotope particulier, en effet, nous a révélé à plusieurs reprises être une niche écologique pour une population thecamoebienne qui semble lui être sinon inféodée, au moins préférencielle.

Plusieurs populations riches nous ont permis d'étudier *C. perlucidus* dans ses manifestations vitales et d'apporter quelques données écologiques sur le périphyton.

Le périphyton

Le périphyton est constitué par le dépôt qui se forme sur les parties immergées des plantes aquatiques ou semi aquatiques. Il consiste en un enduit visqueux généralement verdâtre ou brunâtre, composé principa-

lement de fragments tissulaires de la plante, de Diatomées, d'Algues filamenteuses, de particules minérales et de divers Protozoaires de petite taille.

En ce qui concerne *C. perlucidus*, objet de cette note, ce sont les Phytacées du Lac de Virelles (Prov. du Hainaut) et les Carex des étangs des Epioux (Ardenne), qui nous ont montré les populations les plus nombreuses et les plus stables de cette intéressante espèce.

Matériel et méthode

Le périphyton que nous avons étudié provient de différentes plantes appartenant aux genres: *Potamogeton*, *Typha*, *Sparganium* et *Carex* de différents points d'eau en Belgique, tous ont montré des biocénoses sensiblement semblables. Le périphyton est obtenu par raclage de la partie immergée des plantes aquatiques, il est ensuite immédiatement placé dans l'eau distillée en flacons.

Au laboratoire, après agitation énergique, il est filtré sur tamis de 0,25 mm et réparti en boîtes de Petri. Les Thecamoebiens sont prélevés à la micropipette et rassemblés sur lames sous couvre-objet suivant la méthode simple de Maupas (1888). Pour prolonger les observations, les lames sont conservées en chambre humide. Cette méthode permet la survie des Thecamoebiens pendant quelques jours. Afin d'analyser la répartition des espèces, quelques plantes ont été retirées de l'eau et les racines ainsi que la base des tiges ont été étudiées séparément.

Observations

Rappel de diagnose

La thèque de *C. perlucidus* présente les caractères du genre *Cyphoderia*, la section transversale est circulaire. Le col recourbé forme un angle variant de 30 à 45° avec l'axe longitudinal, le pseudostome est circulaire, le bord simple peut être très légèrement évasé chez certains individus.

Le cytoplasme très hyalin n'emplit que les 2/3 de la thèque, les épipodes sont rares, une ou deux ou deux pulsoles sont visibles, il contient peu d'éléments figurés, seules quelques granulations de formes irrégulières sont généralement massées au niveau du col. La région postérieure du cytoplasme est toujours extrêmement claire et limpide.

Le noyau sphérique mesure 7 µm, il contient des nucléoles en nombre variable. Les pseudopodes de type "Filosa" sont très fins et souvent très longs, non anastomosés (Fig. 1 4). La forme générale de la thèque varie légèrement suivant la localité (Pl. I 5-11).

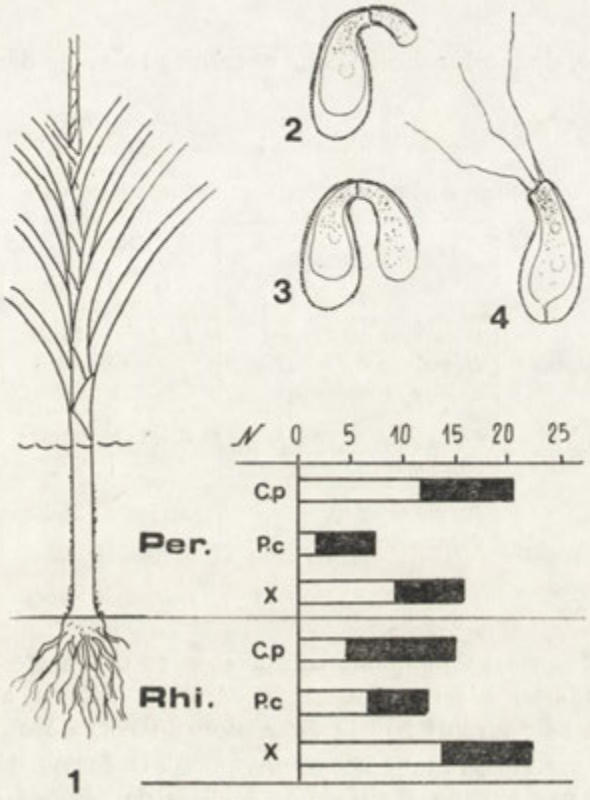


Fig. 1 *1-4*, *1* — Histograme des répartitions (Zones noires — espèces actives, zones blanches — thèques vides, Per — périphyton, Rhi — rhisosphère, C.p — *Cyphoderia perlucidus*, P.c — *Paulinella chromathophora*, X — *Trinema* et *Euglypha*), *2-3* — Deux phases de la reproduction, *4* — Emissions pseudopodiques

Structure

Comme chez toutes les *Cyphoderia*, la structure de la thèque est contituée d'un base organique incrustant des éléments circulaires siliceux suivant le même schéma de disposition classique à ce genre. Observées à fort grossissement, les thèques vides montrent que chaque écaille est bordée par un léger renflement chitinoïde qui en souligne les contours, ce qui les fait apparaître moins régulièrement circulaires.

En dissolvant le vernis organique par l'acide sulfurique concentré, on constate que ces éléments siliceux sont en réalité des écailles plates biconvexes qui ne sont pas soudées entre elles par des points de contact comme le décrit P e n a r d (1902) pour *C. ampulla*, mais indépendantes les unes des autres (Pl. I 12, 13). Ces écailles ont un diamètre variant de 0.25 à 0,50 μm et une épaisseur au centre deux fois moindre. Toutes ne sont pas parfaitement circulaires et certains individus montrent des écailles assez irrégulières suivant les localités.

Dimensions

Le tableau 1, donne les dimensions extrêmes relevées dans différentes localités.

Tableau 1

Dimensions extrêmes relevées dans différentes localités

Localites	Plantes	Dimension en µm		
		L	D	Ps
Ille Edgeøya	*Distichium capillaceum* *Homalothecum nitens* *Calliergon giganteum*	50–58	25–29	10–11
Etang des Epioux	*Carex* sp. *Sparganiu* sp.	40–51	22–31	10–11
Lac de Virelles	*Potamogeron* sp. *Phyta* sp.	50–52	22–30	10–12

Reproduction

La reproduction est également conforme à ce qui à été observé chez d'autres *Cyphoderia*. L'arrangement des écailles débute au niveau du pseudostome et se poursuit par la formation du col, alors qu'une partie du cytoplasme exuvié prenant progressivement la forme définitive n'est encore protégé par aucune écaille. Les écailles de réserve existent dans le cytoplasme, mais elles sont pratiquement invisibles en raison de leur indice de réfraction élevé.

Nous avons observé deux stades de la reproduction (Fig. 1 *2*, *3*), 18 minutes séparant ces deux figures.

Répartition des espèces sur les plantes

Le périphyton de la base des plantes immergèes, la rhizosphère de leurs racines et la vase qui les entourent, forment un ensemble propice au développement de nombreuses espèces de Thecamoebiens.

La population la plus fréquente se compose des espèces suivantes: *Paulinella chromathophora* Lauterborn (Pl. I 14), *Trinema lineare* Penard, *Trinema penardi* Thomas et Chardez, *Trinema complanatum* Penard, *Euglypha loevis* Perty, *Euglypha rotunda* Wailes et *Arcella hemisphaerica* Perty. Cette liste n'est pas exhaustive, nous ne citons que les espèces plus habituellement rencontrées. Ces quelques espèces fréquentes sur la base des plantes immergées sont toutes de petites tailles et concordent avec les analyses de Schönborn (1962), en effet, la principale caractéristique de cette faunule étant leur taille réduite.

En étudiant leur répartition sur les Typha du Lac de Virelles, nous avons compté le nombre de thèque présentes dans les différentes zones sur un ensemble de cinq plantes, ce qui nous permet de dresser l'histograme (Fig. 1 *1*). Nous constatons qu'il existe une certaine intercontamination entre ces zones, en particulier en ce qui concerne le nombre d'espèces actives (figurées en noire), ce qui corrobore également les observations de Schönborn (1967) sur l'influence que peut avoir le périphyton sur les populations sédimentaires avoisinantes.

Pour *C. perlucidus*, les chiffres montrent que le milieu le plus favorable est le périphyton, alors que pour les *Trinema* et les *Euglypha*, il s'agit de la rhizosphère et le sapropèle qui l'entoure.

Conclusion

Le périphyton est un milieu particulier, qui abrite une microfaune extrêmement importante et diverse. L'activité bactérienne y est intense. Parmi les Protozoaires Ciliés, les Peritriches sessiles sont toujours extrêmement nombreux: *Vorticella*, *Carchesium*, *Epistylis*, *Opercularia*, ainsi que des Hypotriches de petites tailles, diverses espèces de *Litonotus* et des Gymnamoebiens.

La composition des populations de Protistes du périphyton, mériterait un recensement faunique beaucoup plus complet.

De nombreuses espèces sont également présentes dans le sapropèle de la base des plantes aquatiques, ou se retrouvent toujours de nombreuses thèques vides.

RÉFÉRENCES

Beyens L. and Chardez D. 1986: Some new and rare testate Amoeba from the Arctic. Acta Protozool., 25, 81-91.

Maupas E. 1888: Recherches expérimentales sur la multiplication des infusoires ciliés. Arch. Zool. Exp. Gén., 2, 185-277.

Penard E. 1902: Faune Rhizopodique du Bassin du Leman. Kündig Edit., Genève.

Schönborn W. 1962: Die Okologie der Testaceen im oligotrophen See, dargestellt am Beispiel des Grossen Stechlinsees. Limnologica, 1, 111-182.

Schönborn W. 1987: Taxozönotik der beschatten Süsswasser Rhizopoden. Eine raumstrukturanalytische Unteruschung über Lebensraumerweiterung und Evolution bei der Mikrofauna. Limnologica, 5, 159-207.

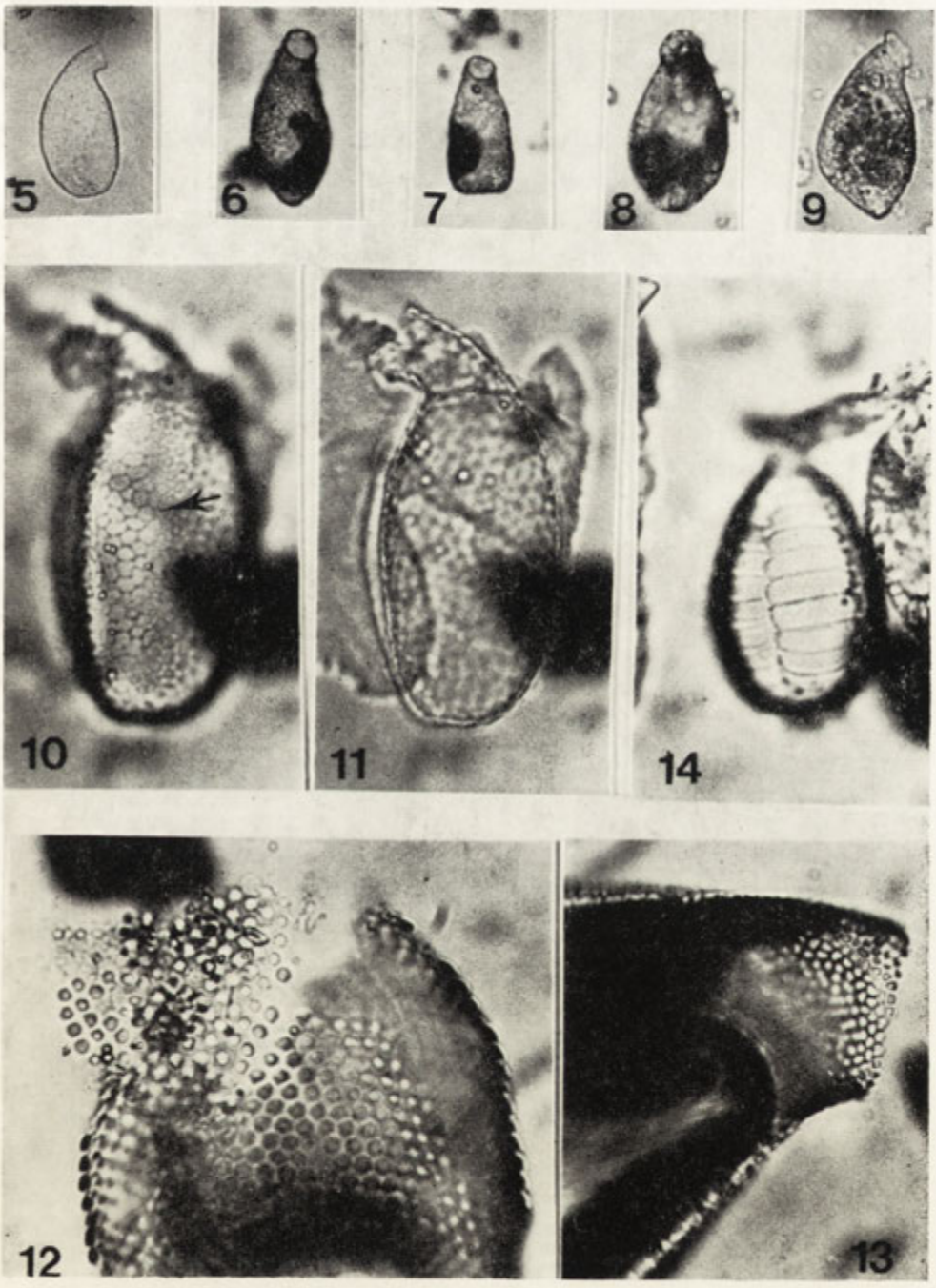
SUMMARY

In this work, we study the periphyton of aquatic plants, considered as particular ecologic nook.

We bring a few precisions about the biology of *Cyphoderia perlucidus* and of kinds that usually come with it.

EXPLICATION DE PLANCHE I

5-13 *Cyphoderia perlucidus*
5-9: Quelques variantes morphologiques (× 400)
10: *C perlucidus* (mise au point sur la face inférieure, la flèche indique la forme réelle des écailles)
11: Même spécimen (mise au point sur la face supérieure l'aspect des écailles est différent) × 630)
12: Thèque détruite par H_2SO_4, les écailles sont isolées (× 1000)
13: Détail du pseudostome (× 1000)
14: *Paulinella chromathophora* (× 630)



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Eimeria spp. of Roe Deer (*Capreolus capreolus* L.) in an Apenninic Area of Italy

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Synopsis. Examination for coccidia of 133 roe deer (*Capreolus capreolus*) faecal samples from an Apenninic area in Florence Province revealed the following 4 species: *Eimeria capreoli* Galli Valerio, 1927 (in 14% of the specimens), *E. panda* Supperer and Kutzer, 1961 (26%), *E. ponderosa* Wetzel, 1942 (16%), *E. rotunda* Pellérdy, 1955 (1.5%). Morphological descriptions of these species are given. Sporulation of oocysts was obtained using an apparatus for continuous oxigenation studied by one of the authors (P. G.). An experimental transmission of *E. panda* was performed to a male roe deer, and of *E. capreoli*, *E. ponderosa* and *E. rotunda* to a female roe deer.

Roe deer (*Capreolus capreolus*) is a naturally spreading species and its population is increasing both in Alpine and Apenninic areas of Italy. This characteristic makes it expecially interesting from ecologic — naturalistic, and also from economic — hunting points of view. The aim of the present study is to extend the knowledge on the parasites of this ruminant, particularly on the coccidia. The coccidia of roe deer were previously studied in Italy only by Mantovani et al. (1970).

Material and Methods

During the period from September 1987 to January 1988, 133 faecal samples of roe deer were collected from an Apenninic area in the province of Florence

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Fig. 1. Area of survey

(Fig. 1) highly populated by this animal. It is a mountain area (600-1500 m above sea level) partially cultivated and partially woody by chestnut trees, conifers and other deciduous trees broken up by bushes. To collect only fresh faeces, many animal groups (Fig. 2) were followed by ambush. The samples were examined after flotation in saccharize solution of specific gravity 1.3. The highly positive samples were diluted in 2.5% potassium dichromate solution and incubated at 25°C in an apparatus (Fig. 3) for continuous oxigenation, realized by one of the authors (G. P.). This tool is made by a large glass jar (3 l) airtight plugged. An aquarium pump is provided for a continuous soft blow of atmospheric air into the medium. The pressure increasing is avoided by an exhaust pipe on the plug top. The morphology of the oocysts was studied with a binocular microscope equipped with a 100 × objective and a 10 × ocular micrometer.

A male and a female roe deer, 32 month old, negative for coccidia in many previous examinations, were experimentally infected through a stomac tube. The male reveived 7.500 sporulated oocysts of *Eimeria panda*; to the female was given a pool of 47.000 sporulated oocysts of *E. ponderosa* (64%), *E. capreoli* (26%) and *E. rotunda* (10%). To assess the prepatent period of each species, the presence of oocysts was cheked every day after infection. The oocysts collected from wild animals were compared with those obtained from the experimentally infected animals.

Fig. 2. Localities of single faeces collection

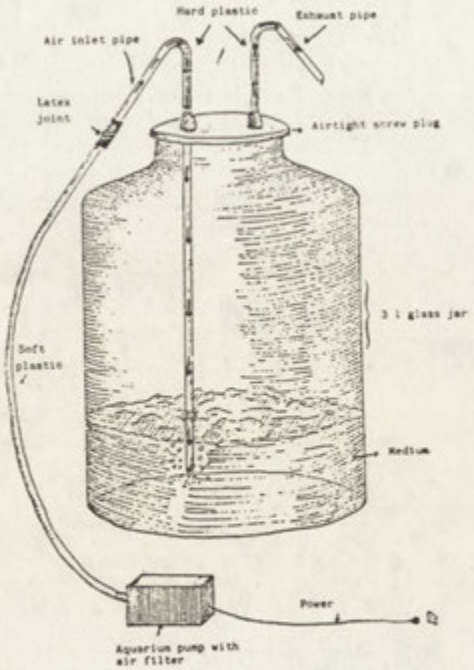


Fig. 3. Apparatus developed for oocysts sporulation

Results

Sixty of 133 examined samples (45%) were found positive for coccidia of the genus *Eimeria*. Four species were identified as follows (all measurements are in μm).

E. panda Supperer et Kutzer, 1961; found in 34 samples (26%).

Oocysts ellipsoidal (Pl. I 4) but slender and often flattened at one side (Pl. I 4b), measuring (n = 99) 31.7 ± 0.16* (27-36) × 16.9 ± 0.12 (14-21) with L/W ratio (n = 99) of 1.88 ± 0.02. Wall smooth, colourless, 1.4 thick, composed of 2 layers; micropyle present, but not easy to observe. The sporont shrinks to form a round mass in the middle of oocyst; oocyst residuum and polar granules absent. Sporocysts (Pl. I 4c) elongated measuring (n = 99) 9.7 ± 0.10 (8-12) × 5.8 ± 0.09 (4-8). Stieda body absent; sporocyst residuum present in form of small granules.

Sporulation took 8 days; prepatent period was 4 days.

E. ponderosa Wetzel, 1942; found in 21 samples (16%)

Oocysts piriform (many oval), bottlenek in shape at the narrower end (Pl. I 5b, d) measuring (n = 106) 38.6 ± 0.20 (35-43) × 26.3 ± 0.18 (22-30) with L/W ratio of (n = 106) 1.48 ± 0.01. Wall dark, wrinkled (Pl. I 5a), double layered, 2.8 thick. Micropyle present. The sporont is large and spherical. Oocyst residuum and polar granules absent. Sporocysts elongated (Pl. I 5c), lemon shaped, measuring (n = 106) 14.9 ± 0.19 (10-22) × 8.2 ± 0.09 (6-10). Stieda body and sporocyst residuum present.

Sporulation time and prepatent period were 16 days, each.

E. capreoli Galli Valerio, 1927; found in 18 samples (14%)

Oocysts ovoid or pyriform (Pl. II 6a, b), measuring (n = 110) 35 ± 0.13 (32-37) × 23.7 ± 0.14 (19-26), with L/W ratio of (n = 110) 1.48 ± 0.01. Wall smooth, 1.4 thick and double layered. Micropyle present at the narrower end, but not easy to observe. Oocyst residuum and polar granules absent. Sporocysts ellipsoid and elongated (Pl. II 6c), measuring (n = 110) 14.2 ± 0.12 (11-17) × 7.2 ± 0.09 (5-8). Stieda body and compact sporocysts residuum present.

Sporulation time and prepatent period were 8 days, each.

E. rotunda Pellérdy, 1955; found only in two samples (1.5%)

Oocysts spherical or subspherical (Pl. II 7a), measuring (n = 100) 11.6 ± 0.08 (9.7-11.8) × 10.8 ± 0.08 (9.7-12.4) with L/W ratio of (n = 100) 1.08 ± 0.01. Wall smooth and colourless < 1 thick; micropyle, oocyst resi-

*standard error

duum and polar granules absent. Sporocysts elongated, ovoid (Pl. II 7b), measuring (n = 100) 7.5 ± 0.07 (5.5-8.3) × 3.8 ± 0.07 (2.8-5.5). Stieda body and sporocyst residuum, in the form of small granules, present.

Sporulation time and prepatent period were 5 days, each.

Statistical analysis of size differences between the oocysts obtained from the wild roe deer and from the experimentally infected animals proved not significant ($P > 0.05$).

Discussion

During the study no clinical case of coccidiosis was observed in the roe deer population. In the experimentally infected animals, only in the male (infected with *E. panda*) mucus was seen few days in the faeces dropped all together.

Some morphological differences appeared between the coccidia found in this study and those of the same species described by other authors. The oocysts of *E. panda* present a micropyle not easy to observe. The sporont forms a round mass not at the one end (Mantovani et al. 1970), but constantly in the middle of the oocyst. *E. ponderosa* flotated by the classical method for coccidia (Mantovani et al. 1970, utilized a particular kind of flotation) showed sporocysts of smaller size: 14.9 × 8.2, versus 21 × 9.5 of those described by Mantovani et al. (1970) and 19 × 9.4 described by Wetzel (1942). Oocysts of *E. capreoli* present a micropyle not easy to observe, and oocyst lengths in our study (35) appear higher than those reported by Mantovani et al. (1970), (31.9), by Pellérdy (1955) (30), or by Galli Valerio (1927) (24).

It is our opinion that the differences in the sporulation time observed in our study must be imputed to the use of the described equipment for continuous oxigenation, previously not utilized by other authors.

We found a smaller number of species in the roe deer than other investigators. The prevalence of coccidia in our research (45‰) is similar to that reported by Canestri-Trotti et al. in 1988 (43.6‰) from an area in the province of Forli. However, these authors gave no description of the oocysts found. But the prevalence in their study was higher than in observations of Mantovani et al. in 1970 (32‰). The latter survey was performed many years ago in an Alpine area far from the zone of our descriptions. In accordance with, the evidence provided by Mantovani et al. (1970), Supperer and Kutzer (1961) and by Boch and Lucke (1961), *E. panda* showed the higher prevalence rate. On the contrary, Dyk and Chroust (1973) in Czechoslovakia, and Golemansky (1986) in Bulgaria found *E. superba* and *E. capreoli* as more frequently represented, respectively.

In accordance with data reported by Mantovani et al. (1970) and by Pellérdy (1955) we also did not found coccidia of domestic species.

REFERENCES

Boch J. and Lucke D. 1961: Untersuchungen über Kokzidien des Schalenwildes. Tierarztl. Umsh., 12, 421-425.

Canestri-Trotti G., Scozzoli M. and Testi F. 1988: Indagini parassitologische in caprioli dell'appennino forlivese. Parassitologia, 30 (Suppl. 1), 45-46.

Dyk V. and Chroust K. 1975: The incidence and possible cross transmission of coccidia and helminths in the mouflon and roe deer in Czechoslovakia. Vet. Parasit., 1, 145-150.

Galli Valerio B. 1927: Notes de parasitologie et de technique parasitologique. Zentbl. Bakt. ParasitKde, Abt. I, 103, 177-182.

Golemansky V. G. 1986: Composition, biology and distribution of the Coccidians (*Coccidia, Eimeriidae*) of roe deer (*Capreolus capreolus*) in Bulgaria. Acta Zool. Bulg., 32, 3-9.

Mantovani A., Borrelli D. and Ricci-Bitti G. 1970: On the coccidia of roe deer (*Capreolus capreolus*). Parassitologia, 12, 151-169.

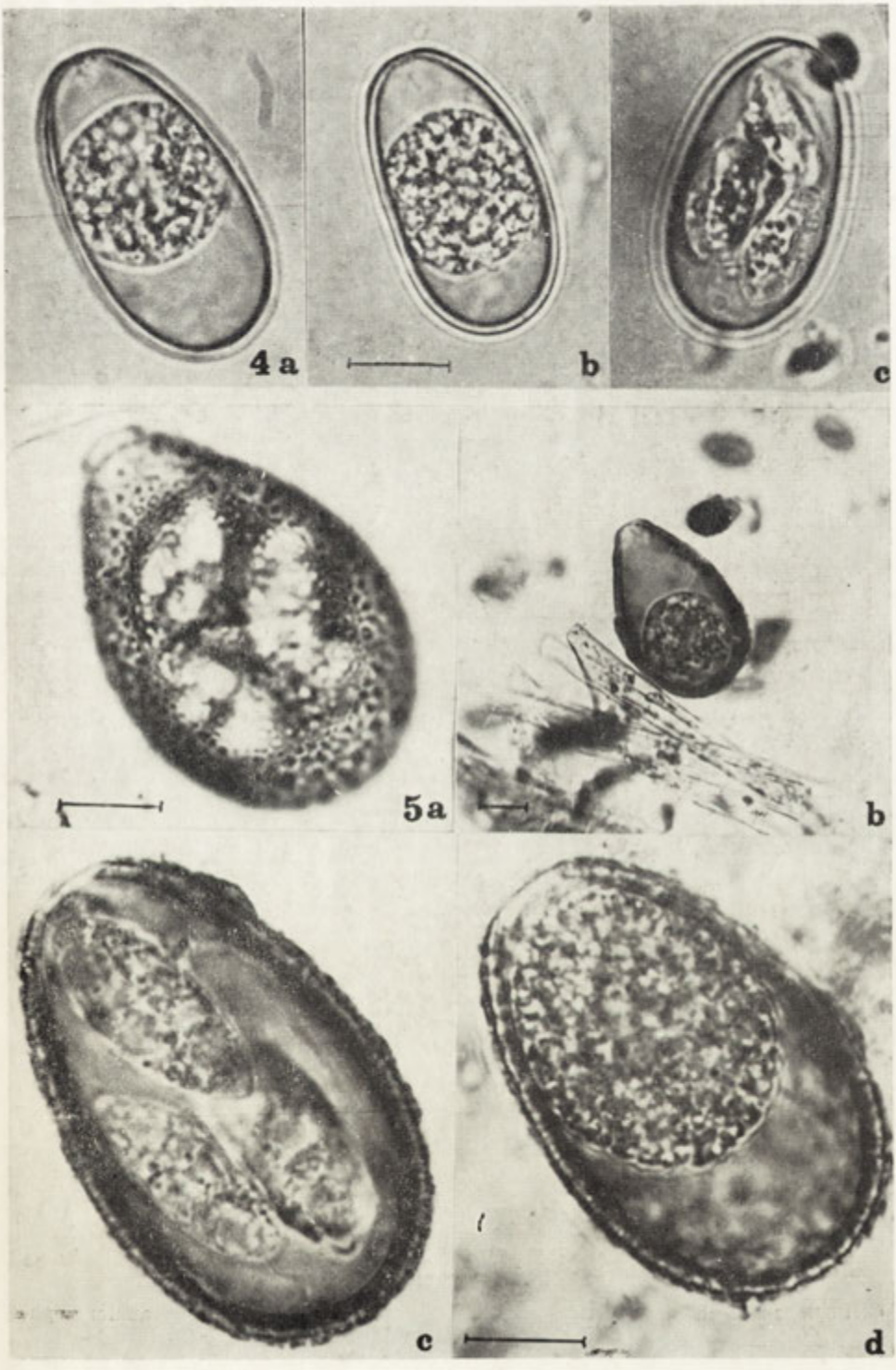
Pellérdy L. 1955 In: Pellérdy L. 1975: Coccidia and Coccidiosis. Akadémiai Kiadó, Budapest.

Supperer R. and Kutzer E. 1961: Die Kokzidien von Reh, Hirsch und Gemse. Jub. Jahrbuch des O.A.F.W., 128-136.

Wetzel R. 1942: *Eimeria ponderosa* n. sp., ein neues Coccid aus dem Reh. Arch. Tierheilk., 77, 200-203.

EXPLANATION OF PLATES I-II

4: *Eimeria panda*, a, b — usporulated oocysts, c — sporulated oocyst
5: *Eimeria ponderosa*, a — wall roughness, b — piriform unsporulated oocyst, c — sporulated oocyst, d — oval unsporulated oocyst
6: *Eimeria capreoli*, a, b — unsporulated oocysts, c — sporulated oocyst
7: *Eimeria rotunda*, a — unsporulated oocyst, b — sporulated oocyst
All measurements are in μm. Scale bar 10 μm.



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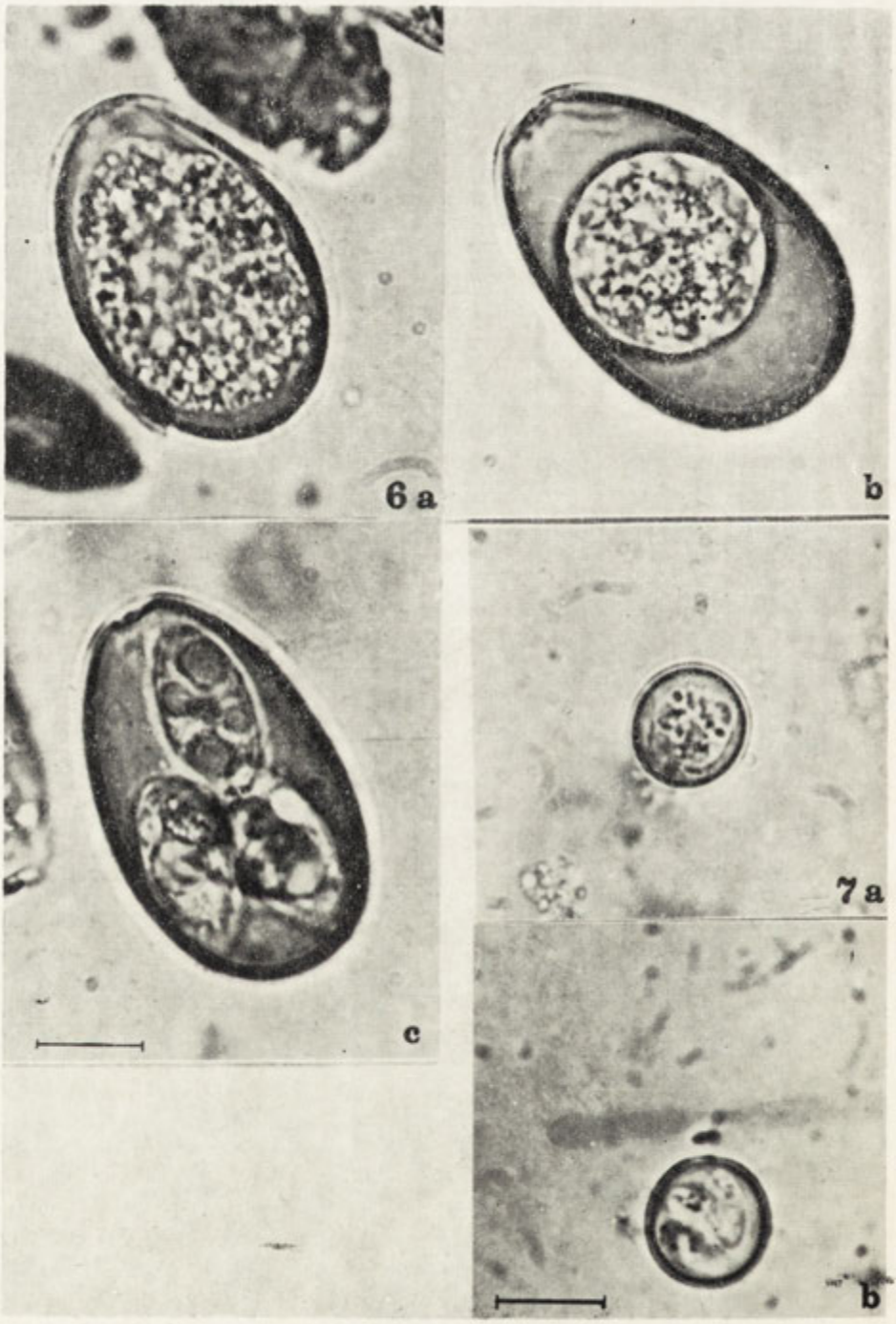

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