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Phenetic and Cladistic Numerical Estimates of Phylogenetic Relationships in *Urostylina* (*Ciliophora*: *Hypotrichida*)

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Synopsis. Several contradictory revisions concerning urostyline hypotrichs have recently been proposed (Borror 1979, Jankowski 1979, Tuffrau 1979, Wicklow 1981, Hemberger 1982, Borror and Wicklow 1983). All these classifications use very limited number of morphological and morphogenetical criteria.

The present attempt applies the numerical taxonomy methods to 19 species (morphotypes) of the suborder *Urostylina*. All genera (with the exception of *Thigmokeronopsis*) are represented. Twenty nine mainly quantitative characters were chosen. The characters pertain to different morphological and morphogenetical aspects of protargol silver-impregnated specimens. Two different types of cluster analysis (UPGMA and PHD) and the Wagner Tree method were used to obtain dendrograms.

The great amount of homoplasy which was estimated by the Wagner Tree method at about 56% is probably the most important result of this study, the more so, as such a possibility has rarely been taken into account by the authors of numerous revisions. This result implies that different states of the majority of characters, including those traditionally considered as having great diagnostic value, may have arisen more than once.

Urostyline hypotrichs have recently been the subject of several revisions (Borror 1979, Corliss 1979, Jankowski 1979, Tuffrau 1979, Wicklow 1981, Hemberger 1982, Borror and Wicklow 1983, Small and Lynn 1985, Tuffrau 1986–1987). Most of these works appeared almost simultaneously but, unfortunately, proposed taxonomies do not agree and are even contradictory in many important issues. All these systems are based on a rather limited number of characters, different authors according different taxonomic significance to morphological, and particularly morphogenetic ones.

The present study is an attempt to apply more objective, numerical taxonomic methods to explain phylogenetic relationships within urostylines. Hitherto, methods

of this kind have not been used in the taxonomy of "lower" hypotrichs. Wicklow's (1981) conception of the suborder *Urostylina* Jankowski, 1979, which corresponds to Borror's (1979) definition of the family *Urostylidae*, was adopted as a starting point.

The taxonomy of hypotrichs above the species level is based on the arrangement of cirri on the ventral surface (Borror 1972). The basic character of the suborder *Urostylina* concerns the way new ciliary structures of the ventral surface are formed. The frontal ciliature of the hypotrichs develops from the fronto-ventro-transverse (FVT) primordium (Borror 1979), which in urostylines has an unusual form. It consists of numerous ciliary streaks disposed obliquely to the long axis of the cell. Their subsequent condensation and migration result in a complex pattern of different categories of cirri (Fig. 1). Most of the streaks differentiate into two cirri each, giving rise to a characteristic zig-zag sequence of midventral cirri (MV). The presence of this double row of cirri is a diagnostic character of *Urostylina* (Wicklow 1981). The streaks closest to paroral membranelles give rise to frontal (FC),

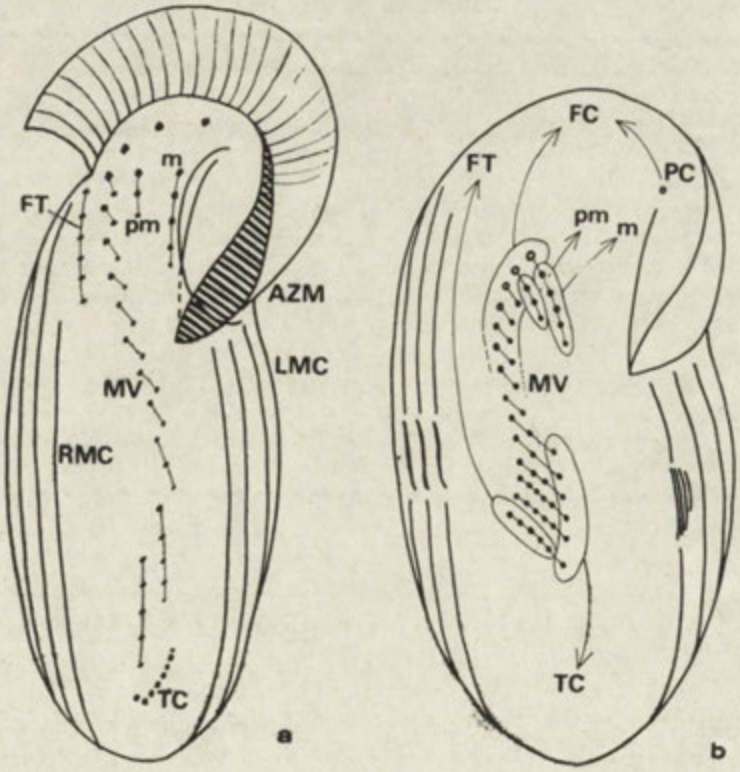

Fig. 1. General pattern of ciliary structures in the suborder *Urostylina* (schematic representation). a — Arrangement of cirri on the ventral surface of an interphase specimen; b — development and migration of different categories of cirri during morphogenesis. Most of the structures develop from a distinct fronto-ventro-transverse (FVT) primordium which occupies the central place of the scheme (details in the text)

malar (m), and in some species also paramalar (pm) cirri (Borror and Wicklow 1983). Some streaks differentiate into more then two cirri, and in some species MV in the posterior half of the cell are arranged in oblique or longitudinal series. Two or more anteriormost cirri originating from the last streak become fronto-terminal or migratory (FT) cirri. The posteriormost cirri in some streaks develop into transverse cirri (TC). Differentiations of this basic developmental scheme lead to diverse morphotypes within the suborder (Borror and Wicklow 1983).

ABBREVIATIONS

AZM adoral zone of membranelles
Dc dorsal cilia
FC frontal cirri
FT fronto-terminal cirri
FVT fronto-ventro-transverse complex
IPM inner paroral membranelle
L length of the cell
LMC left marginal cirri
m malar cirri
Ma macronucleus
MV midventral cirri
OPM uter paroral membranelle
P peristome length
pm paramalar cirri
PC paroral cirrus
RMC right marginal cirri
TC transverse cirri

Material and Methods

Species

The study is based on 19 species (morphotypes) which with one exception were collected by the author (Table 1). The data concerning *Pseudourostyla cristata* were made available by Dr. Jerka-Dziadosz, who kindly offered her preparations. The species were chosen with the intention of covering the widest possible range of different morphotypes of the suborder. All genera belonging to the suborder *Urostylina* (Borror and Wicklow 1983) are represented, with the exception of the monotypical genus *Thigmokeronopsis* Wicklow, 1981. Two more genera, not yet mentioned by Borror and Wicklow (1983), were also considered. These are: *Periholosticha*, Hemberger, 1982 and Keronella, Wiąckowski, 1985. *Holosticha*, the largest and most diverse genus is represented here by five species. Another numerous and controversial group, *Uroleptus–Paruroleptus*, is represented by four morphotypes. The ciliates were collected from various freshwater and terrestrial habitats during the years 1979–1984. Some are new. The formal taxonomic description of one of them was presented elsewhere (Wiąckowski 1985), while further ones are in preparation.

Most of the species were cloned and cultured. Pringsheim solution was used as a basic medium. Some more detailed data about culturing methods are assembled in Table 1. In a few cases culture was not possible and the preparations were made with specimens picked up directly from the sample.

Staining and Measuring

The ciliates were stained with protargol according to the method of Wilbert (1975). A 1:1 (v:v) mixture of Bouin's fluid and saturated mercury chloride was used as fixative. Protargol powder, was shaken directly on the surface of the water containing fixed and cleared ciliates (Wilbert pers. comm.). Traditional stages with oxalic acid and gold chloride (Tuffrau 1967) were omitted. All the species were subjected to exactly the same procedure. The measurements concern protargol stained animals. Ten specimens from each species (with a few exceptions) were drawn with the use of an oil immersion lens (100×) and camera lucida. All necessary measurements were taken with a ruler with accuracy of 1 mm, corresponding to about 0.68 μm.

Table 1

Details of materials used: species, sites of collection, foods applied, and numbers of specimens measured in the study (N)

Species	Author	Collected from	Food applied	N
Bakuella sp.		moss on a tree trunk	*Chilodonella uncinata*	10
Holosticha intermedia	Bergh, 1889	moss on a stream bank	*Chlorogonium* sp.	10
Holosticha multistylata	Kahl, 1932	moss on a calcareous rock	*Chilodonella uncinata*	10
Holosticha muscorum	Kahl, 1932	moss on soil	small *Colpoda* sp.	6
Holosticha polystylatu	Borror and Wicklow, 1983	freshwater aquarium	*Chlorogonium* sp.	10
Holosticha sp.		moss on soil	bacteria	10
Keronella gracilis	Wiąckowski, 1985	moss on a calcareous rock	baker's yeast	10
Paruroleptus caudatas	Stokes, 1886	drainage ditch	*Chlorogonium* sp.	10
Paruroleptus musculus	Kahl, 1932	stream sediment	*Chlorogonium* sp.	10
Periholosticha sp.		moss on soil	*bacteria*	10
Pseudokeronopsis similis	Stokes, 1886	moss on a pond bank	*Chlorogonium* sp.	10
Pseudokeronopsis sp.		pond sediment	diatoms	4
Pseudourostyla cristata	Jerka-Dziadosz, 1964	pond*	bacteria*	3
Pseudourostyla sp.		freshwater aquarium	*Chlorogonium* sp.	10
Uroleptus muscorum	Kahl, 1932	moss on a tree trunk	bacteria + small *Protozoa*	10
Uroleptus sp.		moss on soil	baker's yeast	10
Urostyla grandis (a)	Ehrenberg, 1830	freshwater aquarium	*Colpidium* sp.	10
Urostyla grandis (b)	Ehrenberg, 1830	pond sediment	*Colpidium* sp.	10
Urostyla thompsoni	Jankowski, 1979	brackishwater psammon	bacteria + small *Protozoa*	10

* Data according to Jerka-Dziadosz (1972)

Characters

The characters for analysis were chosen according to the general suggestions of Sneath and Sokal (1973). Various morphological and morphogenetical aspects of the suborder were taken into account. The values of all quantitative characters were transformed into ordered multistate or binary codes. This is a routine procedure which permits characters of different type to be used in the same analysis (Sneath and Sokal 1973). The loss of information caused by coding does not influence significantly the resulting classification. Thorpe (1984) presented several procedures for coding morphometric characters. The whole range of variation of each character is usually cut into several segments in such a way that the boundary lines pass through "natural" discontinuances (dips or gaps in the frequency distribution). The graphic method of Almeida and Bishby (1984), useful in revealing character correlations and reducing the effects of sampling error, was used in this work. The successive segments of the character range were given corresponding

code numbers (0, 1, 2, ...), considered as character states. No evolutionary interpretation (primitive or derived state) was accorded to any particular code. A complete list of the characters with their respective coding is presented below. Symbols pertain to Fig. 1 and 2.

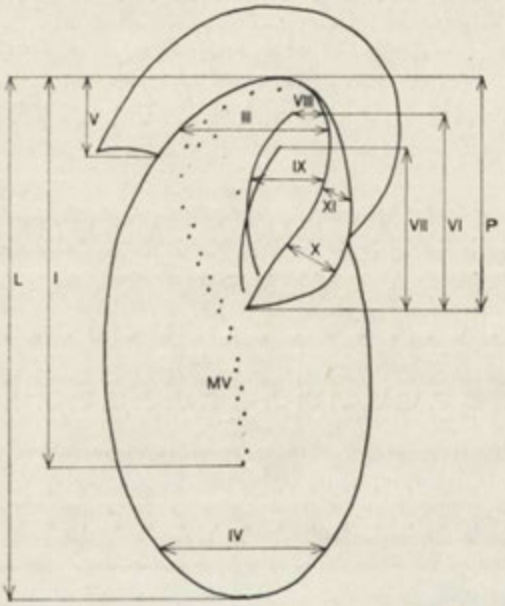

Fig. 2. Measurements made on individual cells

1. Length of the peristome in μm (P): under 41 (0); 42–51 (1); 52–68 (2); over 68 (3)
2. Distance between the anterior end of the cell and the last (posterior) MV cirrus, divided by the length of the cell (I/L): under 0.37 (0); 0.38–0.73 (1); 0.74 and over (2)
3. Cell width measured in one tenth of the cell length and divided by cell length (III/L): 0.16 or under (0); over 0.16 (1)
4. Cell width measured in nine tenths of the cell length, divided by cell length (IV/L): under 0.10 (0); 0.10–0.13 (1); 0.14–0.23 (2); 0.24–0.28 (3); over 0.28 (4)
5. Distance between the anterior end of the cell and the end of AZM, divided by peristome length (V/P): under 0.11 (0); 0.11–0.20 (1); 0.21–0.27 (2); 0.28 and over (3)
6. Distance between the anterior end of the OPM and the cytostome, divided by P (VI/P): 0.57 or under (0); 0.58–0.70 (1); 0.71–0.80 (2); over 0.80 (3)
7. Distance between the anterior end of the IPM and the cytostome, divided by P (VII/P): under 0.66 (0); 0.66–0.71 (1); 0.72–0.77 (2); over 0.77 (3)
8. Maximum distance between OPM and AZM, divided by P (IX/P): 0.23 or under (0); over 0.23 (1)
9. Distance between the anterior end of the OPM and the AZM, divided by the maximum distance OPM–AZM (VIII/IX): 0.69 or under (0); 0.70–0.89 (1); over 0.89 (2)
10. Length of the adoral membranelle situated in the middle of the AZM length, divided by the length of the longest membranelle (XI/X): 0.66 or under (0); 0.67–0.78 (1); over 0.78 (2)
11. Number of the adoral membranelles (AZM): less than 28 (0); 29–42 (1); 43 or more (2)
12. Number of malar cirri (m): 0 (0); 1 (1); 3–6 (2); more than 6 (3)

13. If there is more than 1 malar cirrus, then: number of paramalar cirri (pm): 1 (0); 2–3 (1); 4 or more (2)
14. Number of transverse cirri (TC): 0 (0); 3–5 (1); 6–8 (2); 9 or more (3)
15. Number of fronto-terminal (migratory) cirri (FT): 0 (0); 2 (1); 3–8 (2); more than 8 (3)
16. Number of dorsal kineties: 3 (0); 4–5 (1); 6 or more (2)
17. Number of left marginal rows of cirri (LMC): 1 (0); 2 (1); 3–4 (2); 5 or more (3)
18. If there is more than 1 LMC row, then: each row arises from its own separate primordium (0); all rows are formed in a unique primordium (1)
19. Number of right marginal rows of cirri (RMC): 1 (0); 2 (1); 3 (2); 4 or more (3)
20. If there is more than 1 RMC row, then: each row arises from its own separate primordium (0); all rows are formed in a unique primordium (1)
21. Number of "oblique" midventral rows composed of more than three cirri (arising from a common kinetosomal streak): 0 (0); 1–2 (1): 3–4 (2); 5 and more (3)
22. Number of cirri in the last (posterior) MV row; 2–3 (0); 4–5 (1); 6–17 (2) more than 17 (3)
23. Number of Ma fragments: 2 (0); 5–12 (1); 13–19 (2); 20–79 (3); 80 and more (4)
24. Number of cirri having two following characteristics: i. they are located in the region bounded by the right end of the AZM, ii. they originate from the FVT primordium as the rightmost cirri in the streak: 2 (0); 3–5 (1); 6 and more (2)
25. Dorso-marginal kineties: absent (0); present (1)
26. Mucocysts in the surface layer of the cytoplasm: absent (0); present (1)
27. Localization of the FVT primordium: along the left MV row in the middle of the cell (0); along the left MV row and close to the first transverse cirrus at the same time (1); close to the first transverse cirrus only (2)
28. Caudal cirri (CC): absent (0); present on the end of only one dorsal kinety (1); present on the end of each dorsal kinety (1)
29. If all dorsal kineties have CC, then: there is only one CC on the end of each kinety (0); there is more than one CC on the end of each dorsal kinety (1)

The peristome length (P) is the unique metric character expressed as an absolute value. The choice of the length of the peristome and not the total length of the cell is justified by its smaller variation. The young specimen length is distinctly under the average while its peristome usually equals that of an interphase specimen. The proter frequently inherits the old (unchanged) AZM directly from the parental cell. The remaining measurement characters were used in the form of relations, their values usually being divided by the total (L) or peristomal length (P). The latter was used in the case of most distances measured inside the peristome or in the frontal field.

The complete data matrix is presented in Table 2. Bars indicate no comparison codes resulting from character subordination or (in two cases) the lack of data. The data were scaled before the application of classificatory procedures, i.e., each character state was divided by the range of that character. The result of such scaling is an equal weight given to each character (Colless 1980).

Numerical Methods

The general purpose of phenetic classification is to describe the distributions among organisms of as many of their character states as possible, regardless of their evolutionary relationships (Mc Neill 1978). However, this does not exclude the possibility of later phylogenetic interpretation of the resulting phenogram (Colless 1967). Phenetic methods summarize similarities and differences among organisms without consideration of the number and sequence of changes in characters that have occurred during evolution.

Two different methods of cluster analysis were chosen from the great variety of phenetic procedures. The unweighted pair group method analysis (UPGMA) is the commonly used technique for calculating phenograms. This method generates the least amount of distortion estimated by means of a cophenetic correlation coefficient (Farris 1969 a, Rohlf 1970). The UPGMA phenograms were calculated according to the algorithm of Sokal and Sneath (1963). One of the

Table 2

Character state matrix

Species	Characters					
	1–5	6–10	11–15	16–20	21–25	26–29
Bakuella sp.	12130	32102	12012	00-0-	31400	000-
Holosticha intermedia	12122	10011	11-21	20-0-	00100	020-
Holosticha multistylata	21120	33102	12031	00-0-	00400	010-
Holosticha muscorum	32120	33102	22031	00-0-	00400	000-
Holosticha polystylata	22120	33102	12121	0210-	00400	000-
Holosticha sp.	02121	10011	01-11	10-0-	00100	000-
Keronella gracilis	22141	10021	21-23	10-0-	32320	001-
Paruroleptus caudatus	22001	21102	11-11	00-0-	00001	0020
Paruroleptus musculus	02101	21102	11-11	00-0-	00001	0020
Periholosticha sp.	00001	10022	00-01	00-0-	00200	0020
Pseudokeronopsis similis	32123	00020	21-21	20-0-	00110	100-
Pseudokeronopsis sp.	22023	10010	21-21	20-0-	00310	0-0-
Pseudourostyla cristata	32123	00020	21-30	23131	00320	100-
Pseudourostyla sp.	32123	00020	21-21	21111	00200	100-
Uroleptus muscorum	11011	22102	11-01	10-20	12300	0021
Uroleptus sp.	11021	10021	11-02	10-0-	12300	0021
Urostyla grandis (A)	32121	33102	23230	03130	23410	010-
Urostyla grandis (B)	32121	33102	23230	03030	23410	0-0-
Urostyla thompsoni	01140	21002	01-22	02020	11300	000-

fundamental difficulties in cluster analysis is the choice of an appropriate similarity (or distance) measure. Three different and commonly used functions were chosen: Gower's (1971) coefficient, mean Euclidean distance, and correlation coefficient (Rohlf and Sokal 1965). According to the suggestion of Rohlf (1970), the phenogram with the highest cophenetic correlation was considered as the most credible. A different clustering strategy, PHD (predictive hierarchical dichotomous) based on predictivity, was used as a second phenetic method (Colless 1984). Two phenograms, obtained by different means, were compared by a simple "strict consensus tree" method (Sokal and Rohlf 1962, Rohlf 1982, Smith and Phipps 1984).

Cladistic methods attempt to express similarities and differences in terms of the number and sequence of character changes that have occurred during evolution (Estabrook 1972). Cladograms were calculated by The Rootless Wagner method, and the HTU optimizing procedure was used to increase the parsimony of the trees (Farris 1970). In order to get a better estimate of the minimum length tree, the starting internode was changed systematically in subsequent runs (Colless 1983). More than two passes through the tree were sometimes necessary in the optimizing procedure, before the character states of all hypothetical units were uniquely determined.

The written program does not allow the use of subordinated or missing characters. The estimated Wagner Trees were calculated on the basis of 24 characters (the characters 13, 18, 20, 27, and 29 were omitted). Because the two forms of *Urostyla grandis* differ by only one character, which disappeared in the reduced data set, only one form is represented in the cladogram.

Results

Phenetic analysis. The UPGMA phenogram (Fig. 3), calculated using the correlation coefficient as a similarity measure, provided the highest cophenetic correlation (0.823). The other two phenograms gave distinctly lower values (0.775

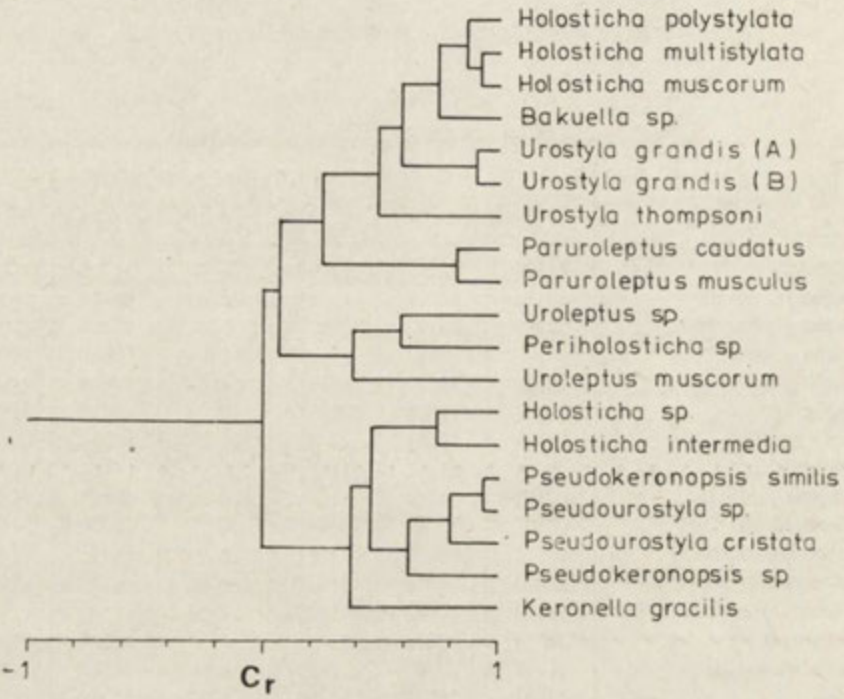

Fig. 3. UPGMA phenogram calculated on the basis of correlation coefficient (C_r) as a similarity measure. (Cophenetic correlation = 0.823)

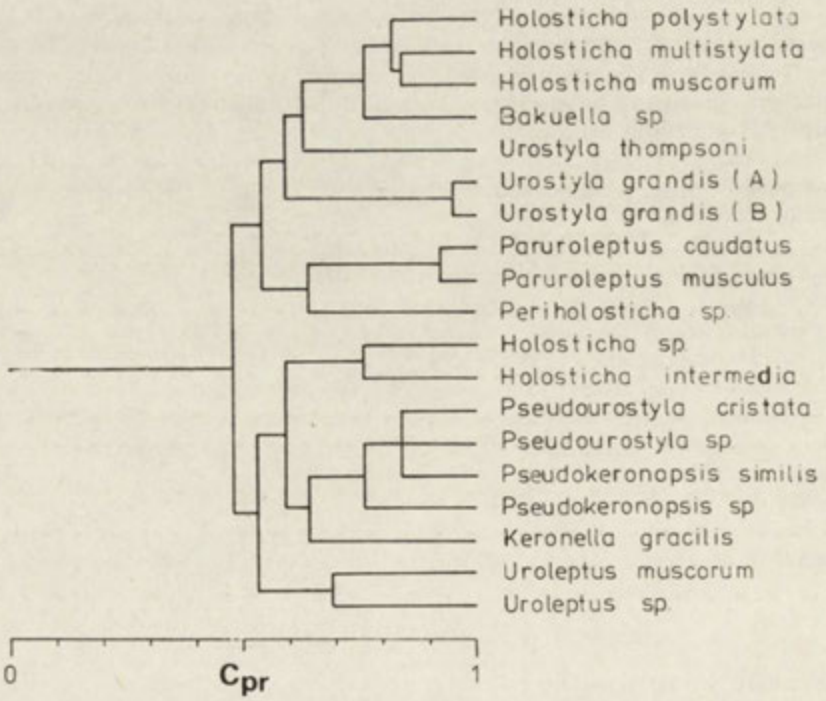

Fig. 4. PHD phenogram; C_{pr} — index of predictivity (Colless 1984)

and 0.752), this being evidence of great distortion with regard to the similarity matrices (Rohlf 1970). These results were omitted. Figure 4 presents a phenogram calculated by the PHD method (Colless 1984). Both dendrograms were calculated on the basis of the complete data matrix (29 characters, 19 species) presented in Table 2.

A consensus tree which summarizes the hierarchical relationships in the two phenograms is presented in Fig. 5. This tree contains only the clusters common to both phenograms. The fact that few species do not enter clusters is evidence that the two dendrograms, calculated by different methods, are in good agreement. Most of the species were distributed between two quite distinct groups. The first one consists of the following species: *Holosticha multistylata*, *H. muscorum*, *H. polystylata*, *Bakuella* sp., *Urostyla grandis*, and *U. thompsoni*. One cannot overlook the striking similarity between the three *Holosticha* species and *Bakuella* sp. The two species of *Urostyla*, *U. grandis* and *U. thompsoni*, do not make a separate cluster. In fact, each of them shows greater numerical affinity with other species (representing diffef rent genera) than with each other. Below are the most typical character states o-this group:

— Both paroral membranelles are long and distinctly curved to the left in their anterior fragment.

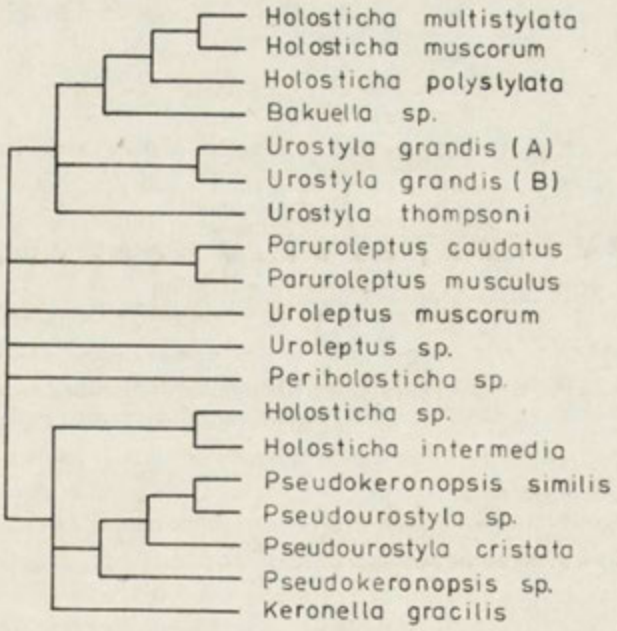

Fig. 5. Strict consensus tree of the two phenograms shown in Fig. 3 and 4. The tree contains only the clusters common to both phenograms

— The right end of the AZM is only slightly prolonged to the right of the cell.

— The longest adoral membranelles are localized at about the middle of the peristome length.

— Three hypertrophied frontal cirri in the region limited by the right end of the AZM (there are more such cirri in *U. grandis*).

— Midventral cirri are shortened or are arranged in some oblique rows in the posterior half of the cell.

— Three dorsal kineties (with the exception of *U. thompsoni*).

— Numerous Ma fragments.

The second group, besides the species of *Pseudokeronopsis*, *Pseudourostyla*, and *Keronella*, contains also *Holosticha intermedia* and *Holosticha* sp. The latter two forms appear to be very distant from the other three *Holosticha* species. Typical characteristics of this group are as follows:

— Paroral membranelles relatively short and not so curved as above.

— AZM distinctly prolonged to the right of the cell.

— The longest adoral membranelles are localized much closer to the cytostome than in the species from the first group.

— No distinctly hypertrophied frontal cirri. Midventral cirri enter the frontal field in the form of two rows curved to the left. As far as this character is concerned, *Holosticha intermedia* and *Holosticha* sp. resemble the species of the first group, but the size differentiation of frontal cirri is almost imperceptible in *H. intermedia*.

— Midventral cirri in the form of a typical zigzag line are prolonged to the transverse cirri (with the exception of *Keronella*, where MV end with some oblique rows).

— Numerous (five of more) dorsal kineties.

— The number of Ma fragments is distinctly smaller than in the first group.

The two species of *Paruroleptus* show greater affinity with the first group, as is seen from the phenograms. The remaining three forms, *Uroleptus muscorum*, *Uroleptus* sp. and *Periholosticha* sp., occupy an indistinct intermediate position.

Cladistic analysis. More then thirty estimated Wagner Trees were calculated, their lenght varying from 54.16 to 60.25 units. The "minimal" length tree is presented in Fig. 6. The "root" is located in the midpoint of the line between the two most distant species. These are, *Urostyla grandis* and *Pseudourostyla cristata*. The length of the branches do not correspond here, as in the case of the phenograms, to taxonomic distances which separate particular species. The tree form depicts only the branching sequence and the resulting hierarchical relationships. The information about the distances between any two taxa is shown in the form of transverse bars. The number of bars corresponds to the number of character changes (evolutionary paths) which occurred along a given branch.

As in the results mentioned above, the estimated Wagner Tree suggests two distinct taxonomic groups. Their composition is very similar to that in the phenograms. The first group, besides *Holosticha multistylata*, *H. muscorum*, *H. polystylata*,

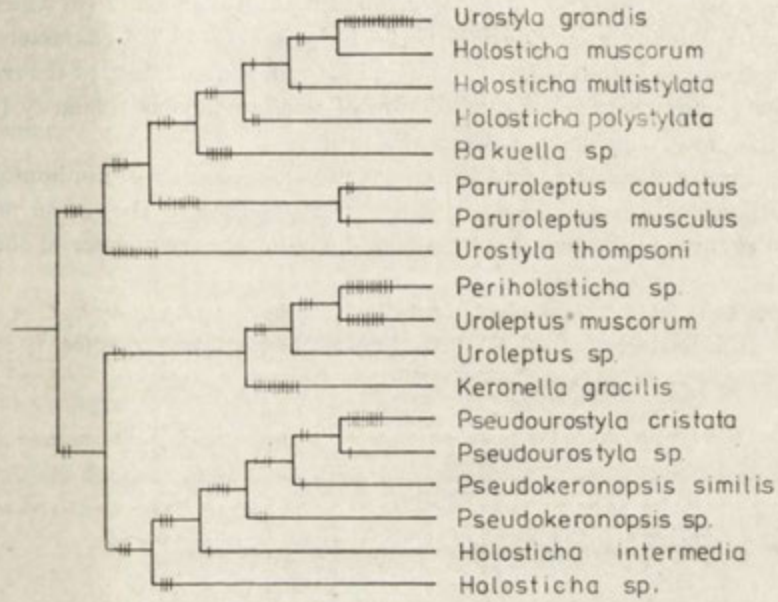

Fig. 6. Estimated Wagner Tree (length — 54.16); the number of transverse bars corresponds to the number of character changes occurring along a given branch

and *Bakuella* sp., contains the two species of *Urostyla*, which are even more distant here. The genus *Paruroleptus* accompanies this group. A distinct group is made up of the species representing the genera *Pseudokeronopsis* and *Pseudourostyla*. As in the phenograms, *Holosticha intermedia* and *Holosticha* sp. show a close affinity with this group and are separated by a great distance from the other three species of *Holosticha*. The remaining forms (the genera *Uroleptus* and *Periholosticha*) whose positions were not clearly defined by the phenetic analysis, together with *Keronella gracilis* are located in a separate, intermediate cluster. All these species have numerous caudal cirri, which beyond this group are to be found only in the genus *Paruroleptus*.

One of the important objections to the method of Wagner Trees is the fact that two trees which differ only insignificantly in their length can have a different topology (Colless 1980). This objection, as well as the fact that one can never be sure if indeed the minimal tree was found, diminishes the credibility of the results. Therefore, analysis is recomended of a few other, slightly longer trees. The length of the next two trees, with regard to the growing length, is 54.83, so they differ from the "minimal" only about 1% of the length. Their topology differs very little from that of the shorter tree shown in Fig. 6. In both trees, the positions of *Urostyla thompsoni* and *Paruroleptus* are interchanged with regard to the shortest tree. In one of the trees also *Holosticha* sp. has a somewhat different position.

The length of the best Wagner Tree estimation found in this study (54.16) is

much longer than that of the "ideal" tree. The length of such a tree (in which each state would appear only once) would be 24 for this data set (24 characters, each with a range equal to unity). The "additional" length is about 56% of the real one, and suggests a very significant contribution of nonhomologous similarity (homoplasy) in the total similarity between the taxa.

Taking the tree from Fig. 6 as a basis, a probable number of nonhomologous changes in each successive character was estimated. Table 3 shows the minimal number of changes in a character (depending directly from the number of character

Table 3

Unit character consistency on the basis of the tree from Fig. 6, for explanations see text

Character	Number of changes		Consistency index
	minimal	real	
12	3	3	1.00
25	1	1	1.00
26	1	1	1.00
6	3	4	0.75
10	2	3	0.67
16	2	3	0.67
5	3	5	0.60
7	3	5	0.60
2	2	4	0.50
8	1	2	0.50
28	2	4	0.50
4	4	9	0.44
14	3	7	0.43
15	3	7	0.43
9	2	5	0.40
23	4	11	0.36
3	1	3	0.33
11	2	6	0.33
22	3	9	0.33
24	2	6	0.33
1	3	10	0.30
17	3	10	0.30
19	3	10	0,30
21	3	10	0.30

states), the real number of changes which occurred in the cladogram, and the unit character consistency coefficient (Farris 1969 b). This index is defined analogously to the notion of the consistency of the data (Kluge and Farris 1969, Farris et al. 1970). It is a character range (i.e., minimal character length) divided by the real patristic length of that character resulting from the actual number of changes in the tree (Farris 1969 b). When characters are coded as discrete states, the patri-

stic length is considered as a number of "evolutionary paths" (Camin and Sokal 1965). If a character is perfectly consistent with a given tree, i.e., each character state appears only once (reversals and convergencies are absent), the value of the consistency index is equal to unity. The more nonhomologous changes are present the greater is the deviation from unity to zero (Farris 1969 b).

Only three out of 24 characters are perfectly consistent with the cladogram (Fig. 6). These are the number of malar cirri, the presence of dorso-marginal kineties, and the presence of mucocysts (characters 12, 25, and 26). Among characters with relatively high consistency index, are localization of the right end of the AZM (5), relative length of the IPM and OPM (6 and 7), relative peristome width (8), AZM form (10), the number of dorsal kineties (16), and the number of caudal cirri (28). Character number 5 is the only one of the above-mentioned characters which has till now been considered important (Borror and Wicklow 1983 p. 113). Conversely, some characters traditionally used in generic diagnoses can be found among those of low consistency, i.e., a narrowing posterior end of the cell (4), and the number of left and right marginal rows (17 and 19).

Discussion

The results presented above make possible new interpretations of certain controversial issues of the urostyline hypotrichs taxonomy.

Pseudokeronopsis

This genus was raised for all those species formerly in *Keronopsis* which have midventral cirri (Borror and Wicklow 1983). *Pseudokeronopsidae* (*Pseudokeronopsis*, *Thigmokeronopsis*) having no hypertrophied frontal cirri are opposed, according to these authors, to *Urostylidae* (*Urostyla*, *Holosticha*, *Bakuella*, *Uroleptus*). However, Jankowski (1979) and Hemberger (1982) suggest that the species which have MV, previously interpreted as *Keronopsis*, should be shifted to *Holosticha*. The results presented in this study (*Pseudokeronopsis* localized always in a distinct cluster than *Urostyla*, *Holosticha*, *Bakuella*, and *Paruroleptus*) support the concept of Borror and Wicklow (1983). The case of *H. intermedia*, which do not follow this scheme, will be discussed below.

Pseudokeronopsis-Holosticha

The position of *Holosticha intermedia* and *Holosticha* sp. in the dendrograms is a somewhat unexpected result. Regardless of the method used, these species are very distant from the other three *Holosticha*, which together with *Urostyla* and *Bakuella* form a distinctly differentiated group. At the same time, they show a close affinity with the *Pseudokeronopsis-Pseudourostyla* group. *Holosticha intermedia* Bergh, 1889 occupies an unquestionable position in the genus *Holosticha*

according to all revisions (owing to the three differentiated frontal cirri). However, a more detailed analysis allows many differences to be discerned with regard to such species as *Holosticha multistylata*, *H. muscorum*, or *H. polystylata*. These are the shape and relative length of paroral membranelles, the shape of the AZM, localization of its right end, the number of malar cirri, and the number of dorsal kineties and Ma fragments. In all these characters *H. intermedia* appears to be more similar to the *Pseudokeronopsis-Pseudourostyla* group. Foissner (1981), describing a similar morphotype (under the name *Holosticha similis*), mentioned numerous mucocysts. In some specimens of the species used in this study, mucocysts could also be noticed. However, as this could not be regularly observed in the same way as in *Pseudourostyla* and *Pseudokeronopsis similis* (in spite of the same staining method), the species was classified as having no mucocysts (at least such as in the other species). Their presence would be an additional character in common with the *Pseudourostyla-Pseudokeronopsis* group. Another character of this category is the kind of nutrition. *Holosticha intermedia*, as well as the species of *Pseudokeronopsis* and *Pseudourostyla*, can best be cultured on *Chlorogonium* sp. while *Holosticha polystylata* is the only species among the opposite group which could be grown on *Chlorogonium* sp. alone (character not used in the analysis).

The results presented above allow the conclusion that the present genus *Holosticha* contains diverse morphotypes on the basis of only one character — presence of at least three differentiated (hypertrophied) frontal cirri (Hemberger 1982, Borror and Wicklow 1983). This is a criterion not precisely defined and sometimes difficult to apply in practice (Dragesco 1966, Jankowski 1979). It is not always clear what different authors mean when they use the term "frontal cirri". Does this term pertain to all cirri in the frontal field, to those anterior to the right end of the AZM, or only to those which are hypertrophied? For example, it seems that the understanding of Kahl (1932 p. 533) is neither the same, as that of Borror and Wicklow (1983 Fig. 1), nor that of Jankowski (1979). According to the last author, all those species of the genus *Keronopsis*, which were included later into the genus *Pseudokeronopsis* by Borror and Wicklow (1983), have no frontal cirri at all (Jankowski 1979, p. 56). For this reason in preparing data for the computer this character had to be substituted by another unequivocally defined one (character 24). It is interesing that the two morphotypes corresponding to *Holosticha intermedia* and *Pseudokeronopsis similis* were placed by Kahl (1932 p. 577) as different varieties of the same species *Keronopsis monilata* (in spite of the apparent differences concerning frontal cirri!).

Holosticha polystylata

The presence of multiple left marginal rows, according to some authors, justifies a separate genus for this species: *Diaxonella trimarginata* (Jankowski 1979), *Trichotaxis pulchra* (Hemberger 1982). The results of the present work, showing

its close affinity with *Holosticha multistylata* and *H. muscorum* (in spite of a different number of LMC rows), support the decision to locate it in the genus *Holosticha*.

Urostyla–Pseudourostyla

The position of *Pseudourostyla cristata* is one of the most important points of disagreement between the results of the present study and those of earlier papers. According to Tuffrau (1979) and Hemberger (1982) *Pseudourostyla* should be considered as a junior synonym of *Urostyla*. They are of the opinion that the morphogenetic differences (Jerka-Dziadosz 1972, Borror 1979) are not sufficient to separate the species with such similar morphology. The results of the present study, however, suggest that there are significant differences between *Urostyla grandis* and *Pseudourostyla cristata*. They are invariably located in quite distinct clusters, regardless of the method (Figs 3–6). *U. grandis* and *P. cristata* even appeared to be the most distant pair of all possible species pairs in the cladogram (Fig. 6). Such a result not only justifies the genus *Pseudourostyla* but perhaps suggests that the two species should be separated at a higher level. The two forms resemble each other in the presence of multiple left and right marginal rows and in their great size. However, a more detailed analysis demonstrates that almost all the other characters used in this study are represented in *U. grandis* and *P. cristata* by distinct or even opposite states: the shape of the AZM, localization of the right end of the AZM, length and shape of both IPM and OPM, frontal cirri organization, number of malar and paramalar cirri, arrangement of the MV in their posterior end, and the number of dorsal kineties.

The number of marginal rows is one of the most important characters traditionally used in the diagnoses of the genera of *Hypotrichida* (Fauré-Fremiet 1961, Corliss 1979).

Pseudourostyla–Pseudokeronopsis

The results of the present work demonstrate a close affinity between species of the two genera. It is interesting that *Pseudokeronopsis similis* is numerically more similar to *Pseudourostyla* than to the other species of its own genus. Live specimens of *Pseudokeronopsis similis* and *Pseudourostyla* sp. were almost indistinguishable. The only character deciding to which of the two genera a given species belongs is the number of marginal rows. Apparently, this may be a false criterion.

Development of marginal systems

Different authors are of extremely opposed opinions as to the taxonomic value of the way of development of marginal rows. The fact, that the entire marginal system develops from one "ventral" primordium in *Pseudourostyla cristata* suggested to Wicklow (1981) that this genus be separated from other urostylines at the level of superfamily. This separation was

maintained by Borror and Wicklow (1983). Tuffrau (1979) and Hemberger (1982) found the character not even sufficient to maintain the genus *Pseudourostyla*. Wiąckowski (1984) found two morphologicaly indistinguishable strains of *Urostyla grandis* morphotypes with different morphogenetical patterns of the left marginal system. In one of them (strain A in the present study) each left marginal row develops from its own primordium while in the other (B) the whole left system develops as in *Pseudourostyla*. This finding is strong evidence against Wicklow's (1981) suggestion. The species distribution on the dendrograms does not allow confirmation of Martin's (1982) interesting hypothesis about the possible evolution of marginal systems in *Hypotrichida*.

Paruroleptus–Uroleptus

Four species with a characteristically narrowing posterior end of the cell were used in this study. They were classified to the genera *Paruroleptus* and *Uroleptus* on the basis of the traditional criterion of Kahl (1932) — the presence and absence of transverse cirri respectively. These species represent quite distinct morphotypes as can be seen in all dendrograms. It seems that they should not be placed in the some genus as proposed by Borror (1972). The narrowing "tail like" posterior end cannot be a sufficient criterion here. The entire *Paruroleptus–Uroleptus* group needs a thorough revision based on protargol stained material. It seems that the presence or absence of transverse cirri will not be the basic criterion for this revision. Probably of much greater importance are the organization of midventral cirri and the presence of dorso-marginal cirri (Martin et al. 1981).

Localization of oral primordium

The place where the first kinetosomes of the new oral structures appear is an important taxonomic character, according to Tuffrau (1969, 1970 and 1972). The formation of the oral primordium in the middle part of the ventral surface should be considered as a primitive character, and close to the first transverse cirrus as a derived one (Tuffrau 1969). Martin (1982) and Martin et al. (1983), taking as basis the hypothesis of the repressive action of the AZM, state that the oral primordium localization can be used as an important character discriminating between higher taxa within *Hypotrichida*.

The results of the present work suggest that Martin's hypothesis, which may be correct on the species level, cannot be used to draw conclusions concerning the phylogenetic relationships between species and higher taxa. *Holosticha* intermedia is till now the only species among *Urostylina* to have the oral primordium localized close to the first transverse cirrus. In the other two species (*H. multistylata* and *U. grandis*) both TC and MV are engaged in the formation of the primordium (Wiąckowski in preparation). No correlation could be seen between oral primordium localization and species morphology as far as the data assembled here are concerned. About half the species used in this study has the AZM relatively greater then *H. intermedia*.

General Conclusions

The significance of the numerical methods used in the present work consists in the indication of similarities which in many cases were "masked" by a single and traditionally important character. In the general opinion, natural classification should describe the distribution among organisms of as many features as possible, and should also permit prediction of the distribution of features not used in the original construction of the classification (McNeill 1978). The systems which arrange species in taxa on the basis of single characters, such as the number of marginal rows, pattern of frontal cirri, or localization of marginal primordia, lead to a situation where very dissimilar species are put together into the same taxon or, conversely, similar forms are to be found in very distant taxa. *Holosticha* according to all the authors, *Urostyla* in the understanding of Tuffrau (1979) and Hemberger (1982), who include *Pseudourostyla* in the same genus, *Uroleptus* according to Borror and Wicklow (1983), are examples of the first situation. A typical example of the second is the localization of *Pseudourostyla cristata* in the system of Borror and Wicklow (1983), or that of *Holosticha polystylata* in a separate genus (Jankowski 1979, Hemberger 1982).

Morphogenetic criteria, as shown in the present work, cannot be very helpful in the consideration of the phylogenetic relationships among the *Urostylina*. Knowledge of the possible variation of morphogenetic patterns and their regulative processes does not permit any serious phylogenetic conclusions. The similar morphogenetic patterns can be observed in quite different morphotypes, while similar or even identical morphologies can result from different morphogenesis (Fleury 1983, Wiąckowski 1984).

In comparing the *Urostylina* species, it can be observed that individual states of different characters are put together in various combinations. This was expressed by the very low consistency of characters with the cladogram. The low values of the cophenetic correlations in cluster analysis are also the result of low consistency. However, the variety of morphotypes in the *Urostylina* seems to be much greater then one might expect. New species, which constitute new and surprising character combinations, are constantly being described (Foissner 1982, Hemberger 1982, Alekperov 1984, Wiąckowski 1985).

The great amount of homoplasy which was estimated by the Wagner tree method at about 56% is probably the most important result of this study, the more so, as such a possibility has rarely been taken into account by the authors of numerous revisions. However, the results of the present study indicate that different states of the majority of characters may have arisen more than once. This concerns also the features traditionally considered as having great diagnostic value, e.g., the frontal cirri pattern, midventral cirri in the form of numerous oblique rows, and multiple marginal rows, which apparently could be subjected to such independent changes as parallelisms, convergencies, or reversals.

Is this result really surprising? As unicellular organisms the ciliates cannot equal multicellular ones in degree of specialization (Levandovski and Corliss 1977). Despite the very fragmentary data, it may be said that urostyline hypotrichs are not very specialized as far as the kind of nutrition is concerned. None of the general morphological types seems to be bound with any particular type of habitat. Very similar forms are to be found in as different habitats as marine psammon, freshwater sediments, soil, or moss. Most of the genera (with the exception of monotypical ones) have their representatives in any one of the cited habitats. The ciliates, with their short generation time and great capacity for new habitat colonization, must have been exposed many times to quite different selective pressures, during their long evolutionary history. Taking into account all the above arguments, one should rather expect a multitude of analogies as a natural feature of ciliate morphology. Taxonomists should exercise great caution in their attempts to reconstruct phylogeny on the basis of morphological characters.

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Protozoa on the Body of *Euphausia superba* Dana from Admiralty Bay (the South Shetland Islands)

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Synopsis. Population of *Euphausia superba* was infested by three forms of epibionts, two of which belong to the family of *Foettingeriidae* and occur most numerously on exopodites and endopodites of thoracic limbs and pleopods. In autumn the infestation was greater than in spring. Molting of krill may have an essential effect on the numbers of epibiotic protozoa.

Among the many papers written on krill only a few concern its epibionts. Stawiszyńska-Janas and Kittel (1983) reported the presence of *Ciliophora-Suctorida* on *E. superba* and *E. crystallorophias* from Admiralty Bay. Rakusa-Suszczewski and Nemoto (in press) described a number of *Suctoria* and *Apostomatidae* forms and their distribution over the body of *E. superba* caught in December and January 1983–1984 in the region of the Indian Ocean. The present work shows the results of a year round observations on the infestation of *E. superba* by *Apostomatidae*.

Materials and Methods

Catches of *Euphausia superba* Dana were done in Admiralty Bay in the periods 3 March 12 May (samples 1 to 13) and 30 September–8 December 1986 (samples 14 to 21). One sample comprised ten individuals taken at random from one catchment obtained in the bay. Size and sex of the examined krill are shown in Fig. 1. Samples collected on the same date (Table 1 and 2) differ by the place of sampling in the bay. In winter Admiralty Bay was frozen, and catchments of krill were thus impossible to make. Single individuals of *E. superba* were analysed by controling the numbers of three forms of protozoa, the occurrence of which on krill had been reported earlier (c.f. Rakusa-Suszczewski and Nemoto, in press). The identified forms (1, 2 and 3) were counted on four exopodites of the first and third pairs of pleopods and also on four exopodites and four endopodites

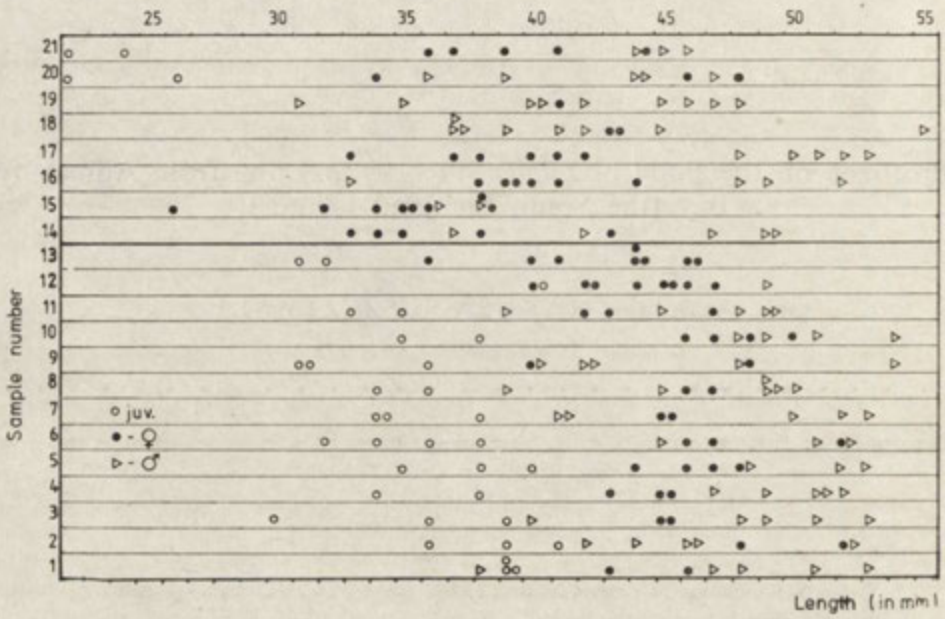

Fig. 1. Sex and size of individuals of *Euphausia superba* Dana used for counting the 3 forms of *Apostomatida* in autumn (3 March 1986 to 12 May 1986, samples 1–13) and spring (30 September 1986 to 8 December 1986, samples 14–21)

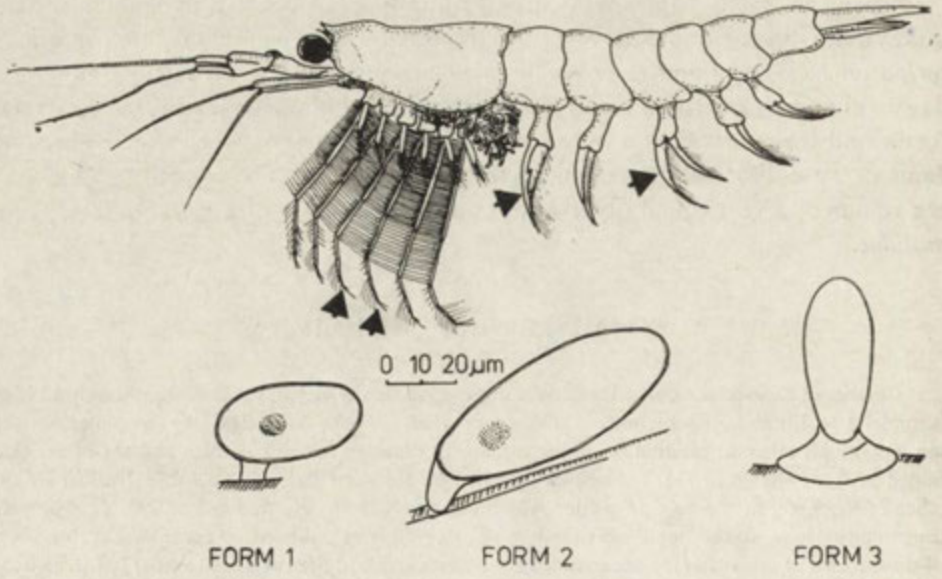

Fig. 2. *Euphausia superba* Dana; Black arrows indicate appendages used for counting Forms 1, 2, 3 of *Apostomatida*

Table 1

Numbers of 1, 2, 3 forms of protozoa: found on selected appendages of *Euphausia superba* Dana from Admiralty Bay (South Shetlands Is.)

Season	Autumn												
Number of sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Date	3.03.86	3.03.86	6.03.86	6.03.86	15.03.86	15.03.86	24.03.86	24.03.86	5.04.86	11.04.86	19.04.86	30.04.86	12.05.86
Forms of protozoa	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
4 exopodites of 1 and 3 pares of pleopods × 10 ind.	89 206 6	58 143 –	51 131 1	43 100 1	24 71 –	20 51 1	23 124 2	17 65 –	19 104 –	70 159 3	14 93 –	74 244 –	30 201 –
4 endopodites of 3 and 4 of thoracic limbs × 10 ind.	1322 – 21	749 6 9	761 – 8	848 – 3	454 – 3	259 – 2	226 1 5	243 – 3	288 – 2	750 – 14	221 – 5	1097 – 10	369 – 5
4 exopodites of 3 and 4 of thoracic limbs × 10 ind.	65 1101 –	40 549 –	22 539 –	37 695 2	14 536 –	25 419 –	17 694 –	22 562 –	24 727 1	44 688 –	45 740 1	56 1490 –	37 1288 –
Total numbers of protozoa	1476 1307 27	847 698 9	834 670 9	928 795 6	492 607 3	304 470 3	266 819 7	282 627 3	331 831 3	864 847 17	307 833 6	1227 1734 10	436 1489 5

Mean values and S.D. Form 1 $\bar{x}$ 661 S.D. 382, Form 2 $\bar{x}$ 902 S.D. 359, Form 3 $\bar{x}$ 8 S.D. 6

Table 2

Numbers of 1, 2, 3 forms of protozoa: found on selected appendages of *Euphausia superba* Dana from Admiralty Bay (South Shetlands Is.)

Season	Spring																							
Number of sample	14			15			16			17			18			19			20			21		
Date	30.09.86			3.10.86			19.10.86			10.11.86.			19.11.86			21.11.86			4.12.86			8.12.86		
Forms of protozoa	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
4 exopodites of 1 and 3 pares of pleopods ×10 ind.	15	164	1	13	134	–	4	38	–	12	193	–	13	163	1	29	176	–	13	192	–	47	385	2
4 endopodites of 3 and 4 of thoracic limbs ×10 ind.	159	10	5	80	–	5	63	–	1	205	–	3	151	–	3	159	1	–	55	–	–	297	–	3
4 exopodites of 3 and 4 of thoracic limbs ×10 ind.	14	519	–	4	265	–	1	162	–	6	554	–	7	583	–	13	651	1	4	683	–	46	1218	2
Total numbers of protozoa	188	693	6	97	399	5	68	200	1	223	747	3	171	746	4	201	828	1	72	875	–	390	1603	7
Mean values and S.D.	Form 1 $\bar{x}$ 176 S.D. 98, Form 2 $\bar{x}$ 761 S.D. 383, Form 3 $\bar{x}$ 3 S.D. 2																							

of the third and forth pairs of thoracic limbs (Fig. 2). The protozoan forms which were found occur also on the remaining 6 exopodites and 10 endopodites of pleopods as well as on 8 exopodites and 8 endopodites of the thoracic limbs of *E. superba*. The appendages chosen for examination (Fig. 2, black arrows) are among those most frequently inhabited by the studied epibionts (c.f. Rakusa-Suszczewski and Nemoto, in press). The numbers obtained by us (Table 1 and 2) are only part of the total numbers (more or less one third) of protozoans which infest krill.

Results

The populations of *Euphausia superba* Dana in Admiralty Bay are colonized by three forms of protozoans, two of which (Forms 1 and 2) belong probably to the family *Foettingeriidae*, order *Apostomatida*, phylum *Ciliophora* (de Puytorac et al. 1984) and represent their phoron stage (resting stage). Form 3 probably also belongs to *Apostomatida*. The examination of *E. superba* individuals in autumn (samples 1 to 13) and spring (samples 14 to 21), Fig. 1, indicates, that krill in all stages of development, regardless of size and sex, were infested by *Apostomatida*. Only the appendages of two among 210 individuals of *E. superba*, were free of the studied epibionts.

Form 1 (Plate I 1, III 4, Fig. 2) 35–45 μm long, 17–22 μm wide occurs most frequently on endopodites of thoracic limbs between the setae, mainly on segments of ischium and merus, and much more seldom on pleopods at the base of setae. This form adheres also to the body of *E. superba* in the area of rostrum, over antennules, antennae, on uropods and telson, in depressions between the segments of appendages.

Form 2 (Plate II 2, III 5, Fig. 2), 75–87 μm long, 25–30 μm wide, always occurs adhering to the setae of exopodites of thoracic limbs and pleopods. In freshly caught krill, some of the protozoans show rotation movements which are visible at the border of plasma and the outer wall of phoron.

In Form 3 (Plate II 3, III 6, Fig. 2), the round base has a diameter of 30–37 μm, body height is 35–50 μm, width 16–20 μm. This forms adheres to setae, appendages and body of krill. On the endopodites of thoracic limbs it occurs on the segments of carpus and dactylus.

Euphausia superba in Admiralty Bay is very numerously infested by forms 1 and 2 (Table 1 and 2). In most samples form 2 was more frequent than form 1. Differences in numbers of forms 1 and 2 on exopodites and endopodites of thoracic limbs and exopodites of pleopods indicate, that there protozoans do not inhabit the same appendages or the same places on one kind of appendages.

In autumn the numbers of forms 1 and 2 found on the body of *E. superba* are greater than in spring (Table 1, 2). The average quantity of form 1 from all samples collected in autumn is more than three times greater than in spring. Form 2 is only slightly less numerous in spring than in autumn. This indicates that there is a seasonal change in the infestation of *E. superba* by *Apostomatida*. Differences in numbers of forms 1 and 2 in consecutive samples in the same season are sometimes higher

than the differences between the average numbers calculated for the periods of autumn and spring. Probably this is the combined effect of the life cycle of the studied protozoan forms and of the molting processes of *E. superba* during growth of these crustaceans.

Discussion

In spite of the great numbers of studies concerning the Antarctic krill, little attention has been given to the presence of krill's epibionts. They were first observed and described by Rakusa-Suszczewski and Nemoto (in press) on the basis of krill samples obtained in the Antarctic, south from Australia. The population of *E. superba* occurring in Admiralty Bay in autumn and spring is being infested by three forms of protozoans (Forms 1, 2, 3). The two first forms are identical with those described from the Indian Ocean and identified as resting stages of species which belong to the family *Foettingeriidae*. Their presence on *E. superba* in the region of the South Shetland Islands points out to the circum-Antarctic distribution of these protozoans and their close association with krill. The places of occurrence of the described forms (1, 2, 3) over the body of *E. superba* are characteristic and they are similar in both studied regions (c.f. Rakusa-Suszczewski and Nemoto, in press). It seems that the process of colonizing of krill by protozoans takes place during krill movements and the filtering of free living ciliated forms of various development stages of *Apostomatidae* (c.f. Chatton and Lwoff 1935). Larger forms (form 2) inhabit in greater numbers the setae of exopodites while smaller forms (Form 1) settle on endopodites. Form 2 colonizes the setae of exopodites on thoracic limbs, and form 1 dwells mainly on segments of endopodites. Endopodites form a filtering basket (Fig. 2) (McClatchie and Boyd 1983) and the net made of setae, as might be supposed, facilitates the settling of free living forms on the appendages of the host. The development cycle of the described forms 1 and 2 of *Foettingeriidae* is not known.

Phoron stage of form 1 is similar to the species *Spirophrya* observed on many crustaceans (Chatton and Lwoff 1935). Form 1 is also encountered in other points of *E. superba* body that is in places of occurrence of microwhirling of water caused by the movements of the animals. Although nearly 100% of individuals of *E. superba* were colonized by forms 1 and 2 both in Admiralty Bay and in the Indian Ocean, the numbers of the protozoans on single individuals of krill were greater in the nearshore area of South Shetlands, than in the open ocean (c.f. Rakusa-Suszczewski and Nemoto, in press).

As reported by Lindley (1978), 3.4% to 16% of north-Atlantic *Euphausiidae* were colonized by *Apostomatida* and similar to our case, greater infestation of the crustaceans was observed in the neashore region. One can expect that the composition of protozoan epibionts and the intensity of their colonizing *E. superba* might be an indicator of the geographical area of origin of a given krill population.

The influence of krill molting on the numbers of infesting protozoa is undoubtful, and the frequency of molts depends on krill age, phase of growth and environmental factors. One can only suppose, that the observed considerable differences in the numbers of protozoans on krill in individual samples (Table 1 and 2) are the consequence of the pre- and post-molting phases of the host. Life cycle of *Foettingeriidae* is tied with the processes of krill molting (Chatton and Lwoff 1935). It is possible that an intensive infestation harms the host. It can be supposed that the protozoans which occur in high numbers on pleopods and thoracic limbs of krill increase the friction of water and thus make it more difficult for the animals to move and to filter food.

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Motor Response of Intact Cells *Fabrea salina* and its Fragments Towards K/Ca Factor in External Medium

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Synopsis. The anterior cell fragments of *Fabrea salina* show towards 50–150 mM KCl longer lasting induced ciliary reversal than posterior fragments. It is shown that higher sensitivity of anterior cell fragments towards potassium ions is due to presence of AZM, which does not exist in posterior fragments. This conclusions is supported by fact, that small anterior cell fragments almost devoid of AZM show similar low sensitivity towards potassium ions like posterior fragments.

The achieved results favour the view that the excitable cell membrane of *Fabrea* show similar physiological differentiation like other ciliates (*Paramecium*, *Stylonychia*, *Dileptus*) as it is evident from differences of response in various groups of cilia towards external stimulation by potassium ions.

Motor behaviour of ciliate protozoa is associated with the action of single or compound cilia which beat in coordinated way obliquely backwards so that the animals swim forward along spiraling line. Ciliates can move in this way rather quickly from one environment to another. It is well known from extensive studies on behaviour and excitability of ciliate protozoa that they may respond to various external stimuli with short or longer lasting depolarization of the cell membrane associated with ciliary reversal (CR) which is due to influx of free external calcium ions throughout voltage sensitive Ca-channels within ciliary cell membrane (Eckert 1972). Potassium ions applied at appropriate concentration in external medium are known as a factor inducing CR by activation of calcium channels within ciliary membrane.

Another important problem is localization of more or less sensitive regions on the cell surface and how it is related to physiological properties of various groups of single and compound cilia. This evoked interest of many authors who were studying motile phenomena in cell fragments of ciliates. Alverdez (1922 a, b) suggested that cilia of anterior region in *Paramecium* are most sensitive to external stimula-

tion, while Parducz (1956) paid special attention to high sensitivity of peristomal cilia.

The existence of anterior-posterior gradient of sensitivity in ciliates was suggested by a number of authors (Doroszewski 1961, Seravin 1962) but this didn't elucidate sufficiently the possible role of various groups of cilia in response of organisms to external stimuli. In this respect hypotrichs proved to be an excellent model as it is evident from more recent studies on *Stylonychia mytilus* (Dryl and Totwen-Nowakowska 1975, Totwen-Nowakowska and Dryl 1976).

The aim of the present study is to analyze the possible role of somatic and peristomal cilia *Fabrea salina* in response towards K/Ca factor in external medium.

Material and Methods

The *Fabrea salina* strain (obtained from the Laboratory of Zoology, Ecole Normale Superieure, Paris) was grown in the medium: 1100 mM NaCl + 130 mM $MgCl_2$ + 70 mM Na_2SO_4 + 25 mM $CaCl_2$ + 22 mM KCl + 5 mM $NaHCO_3$ + 5 mM Tris/HCl (pH 7.2) with an addition of *Aerobacter aerogenes* as standard food supply. Cells used for experimentation were kept in unbacterized medium for 1–2 days. The standard control solution was: 1100 mM NaCl + 130 mM $MgCl_2$ + + 25 mM $CaCl_2$ + 22 mM KCl + 5 mM Tris/HCl (pH 7.2).

In all experimental solutions the ionic strength was kept constant. When the concentration of KCl was increased, the NaCl concentration was decreased by the osmotically equivalent amount. When $MgCl_2$ was extruded from the medium, the $CaCl_2$ concentration was increased. The Mg-devoid, Ca-enriched (−Mg+Ca) medium was: 1100 mM NaCl + 155 mM $CaCl_2$ + 22 mM KCl+ + 5 mM Tris/HCl (pH 7.2).

Direct observations of behaviour were done under low magnification of an optical microscope.

Cell fragments were produced by cutting the ciliate body with a metal micro-scalpel according to pattern shown in Fig. 1. After cutting the ciliates were washed in standard or in (−Mg+Ca) medium. The observations were carried out in KCl containing medium immediately after introducing ciliates to the desired concentration of KCl in standard or (−Mg + Ca) medium.

Electrical recording. Ciliates were penetrated by two microelectrodes (borosilicate capillaries with filament, outer diameter 1 mm) being filled with 3 M KCL. A third microelectrode also filled with 3 M KCl, was placed outside the cell for differential measuring of the membrane potential. The resistance of the microelectrodes was between 20 and 30 MΩ in standard bathing solution. In the voltage − clamp experiments the cell membrane was held at its resting potential in (−Mg + Ca) solution of −13 mV (in standard medium the resting potential was −32±4 mV) (Kubalski 1983) and was depolarized in steps of 10 mV up to +47 mV. The voltage steps were 300 ms in duration. The results presented in Fig. 2 were obtained in the Lehrstuhl für Allgemeine Zoologie at the Ruhr-University in Bochum on the set-up described by de Peyer and Machemer (1977) and Deitmer (1984).

Results

Experiments were carried out on intact *Fabrea* and its cell fragments in standard and (−Mg+Ca) medium. The reason to apply the last mentioned medium

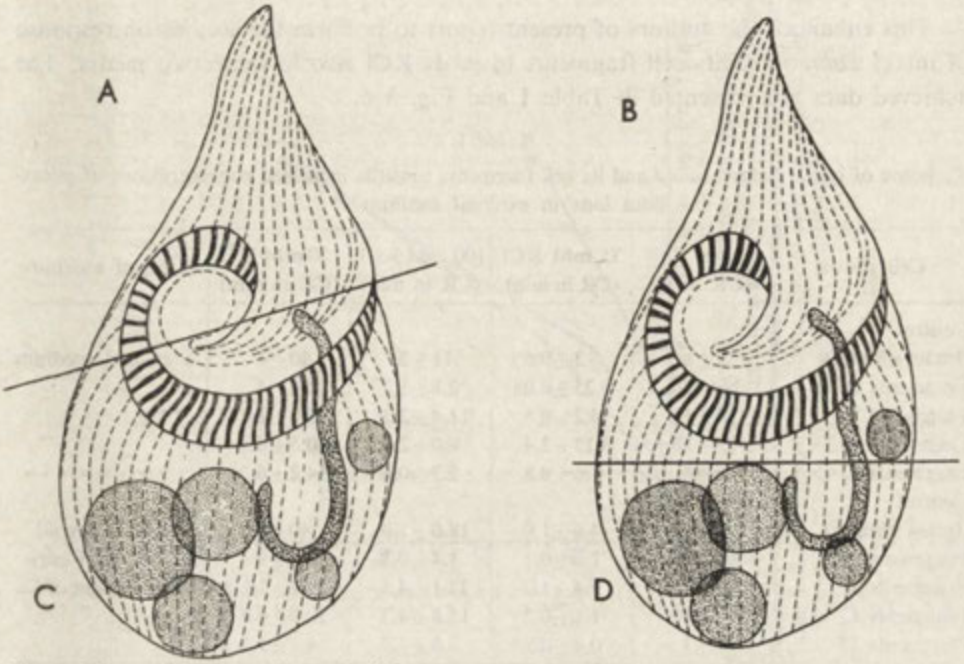

Fig. 1. Schematic presentation of cell fragments *Fabrea salina* produced by cutting according to shown pattern: A — small anterior fragment containing few membranelles of AZM, B — large anterior cell fragment containing whole AZM, C — large posterior cell fragment containing whole AZM, D — small posterior cell fragment devoid of AZM

were the results of preliminary electrophysiological studies which rendered possible the analysis of the early calcium inward current by use of voltage-clamp technique in *Fabrea salina* (Fig. 2) incubated in standard and (−Mg+Ca) medium. It was proved that at all applied voltages the early inward current was lower in standard than in (−Mg+Ca) medium.

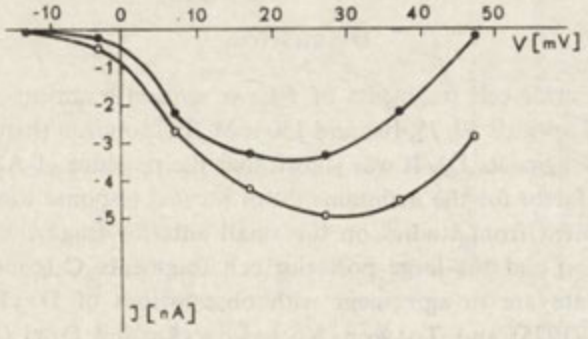

Fig. 2. Changes of the early inward calcium current during voltage clamp analysis in *Fabrea salina*. *1* — data obtained in standard medium (filled circles), *2* — data obtained in (−Mg + Ca) medium (open circles). Each point of the diagram represents an average of 5–6 measurements

This enhanced the authors of present report to perform the studies on response of intact *Fabrea* and its cell fragments towards KCl also in these two media. The achieved data are presented in Table 1 and Fig. 3–6.

Table 1

Response of intact *Fabrea salina* and its cell fragments towards increased concentrations of potassium ions in external medium

Cell status	50 mM KCl (CR in s)	75 mM KCl (CR in min)	100 mM KCl (CR in min)	150 mM KCl (CR in min)	Kind of medium
Control (Intact ciliates)	19±6	3±0.5	11±2	40±4	Standard medium
Fragments A	No CR	0.25±0.08	2.8±1.7	8.8±4	
Fragments B	34±15	4.2±0.8	11.4±2.3	45.3±14.7	
Fragments C	72±15	3.13±2.4	8.0±2.9	40.5±6.3	
Fragments D	20±8	1.6±0.8	2.7±0.6	14.2±6.2	
Control (Intact ciliates)	5.7±2.5	4.6±1.0	18.0±1.0	40±4.0	Medium devoid of Mg with enriched content of Ca
Fragments A	No CR	1.0±0.1	1.4±0.5	4.4±2.1	
Fragments B	53±22	3.4±1.3	22.1±4.5	30.2±10.0	
Fragments C	70±10	1.1±0.2	15.8±4.7	30.0±6.4	
Fragments D	9±3	0.4±0.1	3.8±1.9	4.7±1.3	

Data included in the Table represent duration of K-induced CR on the basis of 10 measurements on average.

Fifty mM KCl induced short-lasting CR (less than 100 s) in cell fragments B,C,D while no CR was observed in fragments A. Higher concentrations of KCl induced in fragments A and D (containing no AZM or only few membranelles) CR of significantly shorter duration than in fragments B and C (containing whole AZM) which showed very similar response to that shown by intact ciliates.

Cell fragments B and C in (−Mg+Ca) medium showed longer lasting CR in response to 100 mM KCl than the same cell fragments in standard medium. Treatment with 150 mM KCl solutions caused rather longer lasting CR in cell fragments B,C. from standard than from (−Mg+Ca) medium.

Discussion

The large anterior cell fragments of *Fabrea salina* (Fragments B) show much higher sensitivity towards 50, 75, 100 and 150 mM KCl solution than small posterior cell fragments (Fragments D). It was shown that the presence of AZM in cell fragment is decisive factor for the maintainance of normal response towards potassium ions as it is evident from studies on the small anterior fragments A (containing few membranelles) and the large posterior cell fragments C (containing most of AZM). These data are in agreement with observations of Dryl and Totwen-Nowakowska (1975) and Totwen-Nowakowska and Dryl (1976) on single and double animals of *Stylonychia mytilus* in which the anterior-posterior polarization of excitability is clearly shown by the high sensitivity towards potassium

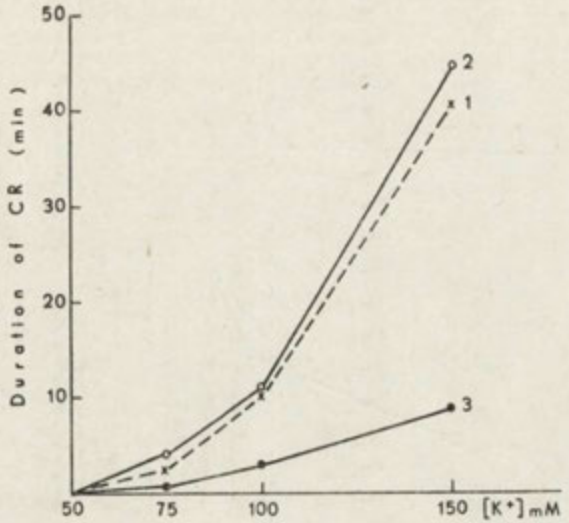

Fig. 3. Response of intact cells (*1*) and anterior cell fragments (*2*, *3*) *Fabrea salina* towards various concentrations of KCl in standard medium. Duration of induced CR is presented by mean values from data included in Table 1. *2* — corresponds to behaviour of fragment A, *3* — corresponds to behaviour of fragment B

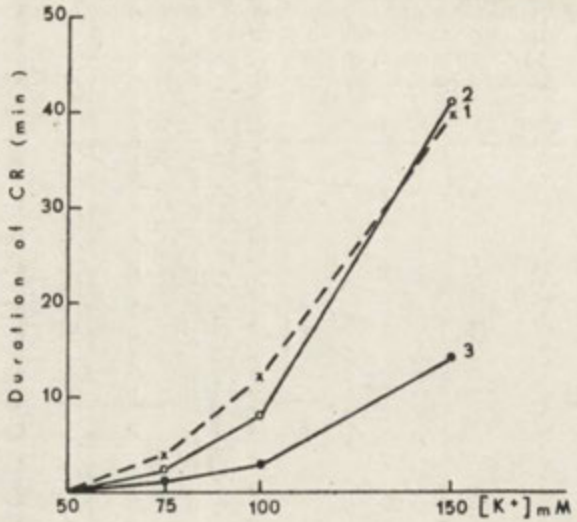

Fig. 4. Response of intact cells (*1*) and posterior cell fragments (*2*, *3*) *Fabrea salina* towards various concentrations of KCl in standard medium. Duration of induced CR is presented by mean values from data included in Table 1. *2* — corresponds to behaviour of cell fragment C, *3* — corresponds to behaviour of cell fragment D

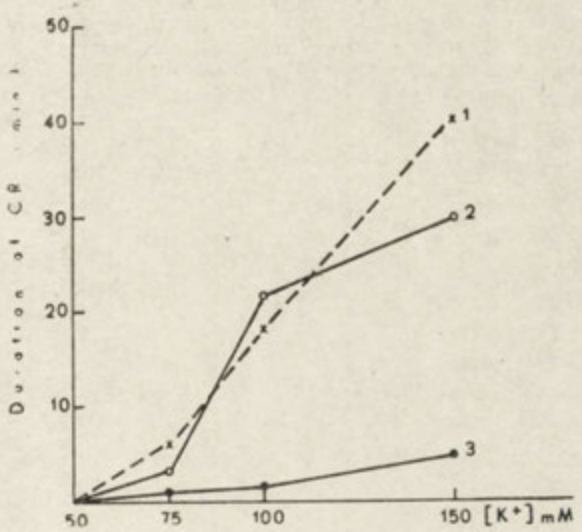

Fig. 5. Response of intact cells (*1*) and anterior cell fragments (*2*, *3*) of *Fabrea salina* towards various concentrations of KCl in (−Mg + Ca) medium. Duration of CR is presented by mean values from data included in Table 1. *2* — corresponds to behaviour of cell fragment A, *3* — corresponds to behaviour of cell fragment B

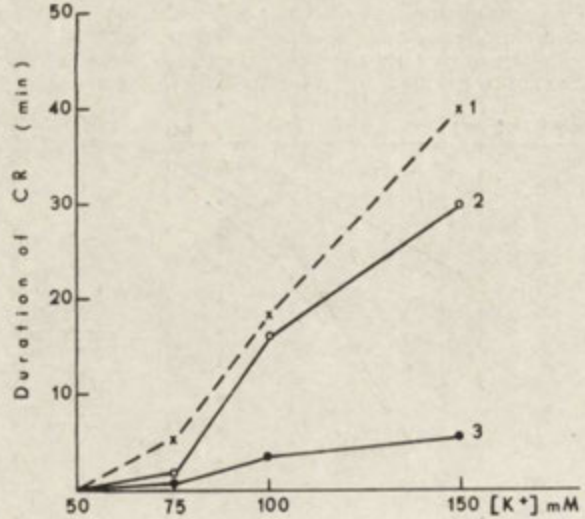

Fig. 6. Response of intact cells (*1*) and posterior cell fragments (*2*, *3*) of *Fabrea salina* towards various concentrations of KCl in (−Mg + Ca) medium. Duration of CR is presented by mean values from data included in Table 1. *2* — corresponds to behaviour of cell fragment C, *3* — corresponds to behaviour of cell fragment D

ions of the anterior cell fragments (containing AZM and frontal cirri) when compared with low sensitivity of the posterior cell fragments. Recent electrophysiological studies (Deitmer et al. 1983, Deitmer 1984) correspond well with the above mentioned findings. They showed that two components of the action potential discovered in *Stylonychia* (the first — fast and graded, the second — prolonged and "all or none") probably correspond to two types of Ca inward currents (the first — small, and the second — large), which are separable by their localization in the membrane. It appeared that the smaller Ca current and the "all or none" component of the action potential were associated with the presence of AZM. It is worth to underline once more in this connection that behavioural studies reported in this article brought evidence that the intact cells and the fragments of the cell containing AZM showed longer response to potassium ions than those devoid of AZM. This fact was even better visible in external medium devoid of Mg, but enriched in Ca, when the early inward current in *Fabrea* was increased. In (−Mg+Ca) solution the process of restoration of forward ciliary movement is much faster in the fragments devoid of AZM comparing the intact cells and the cell fragments containing AZM but also in a comparison to the analogous fragments in standard medium. At present it is not known whether different types of Ca inward current exist in *Fabrea* ciliary membrane — nevertheless the achieved data may indicate that a slower relaxation of Ca-dependent ciliary reversal in the fragments containing AZM is due to slower inactivation of Ca current and thus a different Ca conductance may exist in these cell fragments.

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Physiological Adaptation of Starved *Tetrahymena pyriformis* GL to Colistin

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Synopsis. The effect of starvation upon the ability of oral morphogenesis recovery in *Tetrahymena pyriformis* GL treated with colistin was studied. The capacity for recovery, i.e., predivision stomatogenesis, oral replacement and superseding predivision stomatogenesis by oral replacement depends on the duration of the cells' starvation and their exposure to the antibiotic as well as the concentration of the latter in the culture. It has been found that in spite of the fact that autophagy, is activated, *Tetrahymena* is capable of oral morphogenesis recovery within the initial 20 h of starvation. After prolonged starvation, the capacity gradually declines and after approximately 30 h of starvation the cells are unable to reactivate the process of recovery at higher concentrations of the antibiotic.

Some ciliate species are capable of adaptation to the continuous presence of cell metabolism inhibitors contained in non-lethal concentrations in the medium. Such cellular response is characteristic on *Tetrahymena* (Frankel 1965, Nelsen 1970, Roberts and Orias 1974, Szablewski 1984, 1985) and on *Chilodonella* (Kiersnowska 1982), and referred to as recovery (Frankel 1965). The reaction, which occurs in all cells, consists of the initial arrest of development followed by reactivation of normal development in spite of the continuous presence of a still active inhibitor in the medium.

In the case of exponentially growing cells the recovery includes the return to the control levels of DNA, RNA and protein biosynthesis (Wang and Hooper 1978) and the renewal of the normal course of morphogenesis (Frankel 1965).

Metabolism in *Tetrahymena* at a stationary phase differs essentially from metabolism in exponentially growing cells (Crockett et al. 1965), this being due among other things, to decreased RNA synthesis (Hallberg and Bruns 1976). In cells derived from the stationary phase of the culture a new kind of stomatogenesis, known as oral replacement (OR), appears beside cells undergoing predivision stomatogenesis (PS) (Frankel and Williams 1973). In such case stoma-

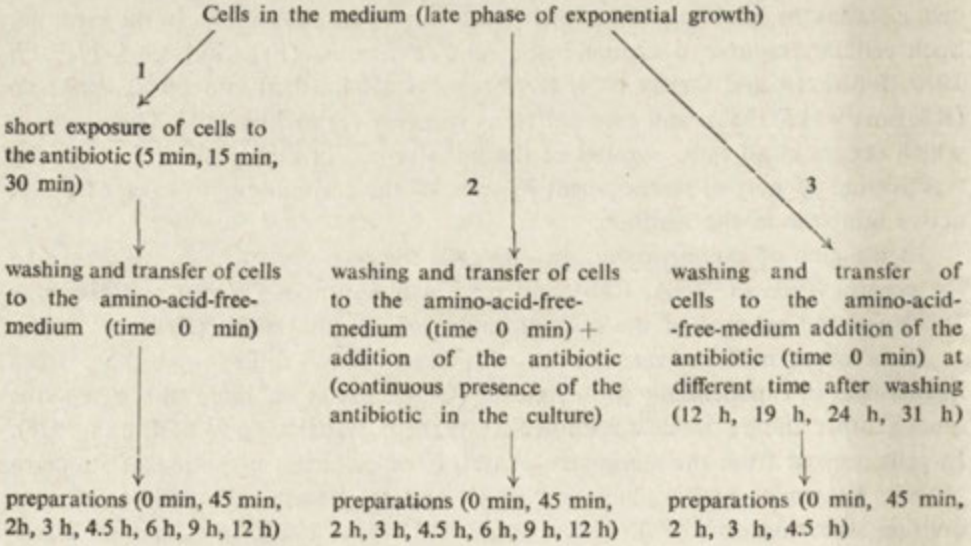
togenesis does not precede cytokinesis. With the duration of the stationary phase the proportions of cells undergoing PS and OR respectively change.

Similar effects may be obtained by transferring the cells into the starvation medium (Kaczanowski 1978, Nelsen 1978). Washing the cells in an amino-acid-free-medium produces a gradual decrease in the number of cells undergoing PS, while the number of cells undergoing OR increases (Frankel 1969, Williams and Nelsen 1973). The course and occurrence of OR may be modified by physiological changes taking place in the cell (Frankel and Williams 1973, Nelsen 1978), physical (Frankel 1964, Gavin 1965, Simpson and Williams 1970) or chemical (Frankel 1970, Nelsen 1970) agents.

Are cells kept in the starvation medium capable of morphogenesis recovery, since autophagy is active under such circumstances (Nilsson 1984)? How long can the starvation last for the cells to be still capable of starting the recovery process? If, following the drug administration, the proportions of cells undergoing the two types of stomatogenesis return to the pre-treatment levels it might be assumed that the processes associated with cell starvation do not inhibit *Tetrahymena* capacity for recovery.

Material and Methods

The study was carried out on the ciliate *Tetrahymena pyriformis* GL. The composition of the medium and conditions of the culture were described in detail in an earlier paper (Szablewski 1985). Twenty four hours after the last inoculation (the exponential growth phase, 10^5 cells/ml), the cells were washed twice in an amino-acid-free medium (Frankel 1965) without bacto-tryptone (Williams and Nelsen 1973). Following the washing procedure, the density of cells per ml was about 5×10^4. Three variants of the experiment were carried out.



The specimens for the experiments were prepared according to the method of Chatton-Lwoff, modified by Frankel and Heckmann (1968). Three hundred ciliates were examined out of each sample.

Simultaneously, the rate of growth of *Tetrahymena pyriformis* GL was investigated in the control culture and following the addition of the antibiotic. The time of washing the cells in the amino-acid-free-medium was referred to as 0 time point. The cell density measured on an electronic cell counter was then ca. 5×10^4 cell /ml. The increase in the cell count (dV) in cultures at a given time point was calculated according to the formula

$$dV = \frac{V_t}{V_0}$$

where V_t – density of the culture at a given time point, V_0 – density of the culture at 0 time point.

In the first four hours of the experiment, beginning at 0 time point, the samples were taken every hour and in the remaining eight hours, every two hours. Between the 12th and 40th h of the experiment, the samples were taken every 12 h.

Colistin, the antibiotic used in the study, was manufactured by the Polfa Pharmaceutical Company at Tarchomin. Following the earlier findings (Szablewski 1981, 1984, 1985), the following concentrations of the antibiotic were employed: 0.05 mM = 0.07 g/l and 1 mM = 1.4 g/l.

The statistical analysis was carried out according to Sokal and Rohlf (1969). All significance was verified with $P<0.05$.

Results

The Rate of Growth of *Tetrahymena pyriformis* GL

In the first hour after the washing of control cells (no antibiotic added) and their transfer to the amino-acid-free-medium, only a slight increase in their number was observed. Subsequently the number of *Tetrahymena* grew with time, doubling within approximately 4 h after 0 time point (Fig. 1). Another doubling of culture density was observed after approximately 8 h and concurrently after that time the dV value gradually decreased. After approximately 12 h no changes were found in the culture density, which remained at the same level until the end of the experiment (Fig. 1).

The lag phase was found to lengthen by about 20 min as compared to the control after addition to the culture of colistin at a concentration of 0.05 mM. Subsequent phases of *Tetrahymena* culture growth in the continuous presence of the antibiotic ran a similar course as in the control culture, while the differences in culture density between the sample in question and the control were not statistically signifcant (Fig. 1).

The continuous presence in the culture of colistin at a concentration of 1 mM significantly affected the rate of cell multiplication. The lag phase lasted ca. 5 h. The first twofold increase in the culture density was observed about 9 h after 0 time point. Twelve hours after 0 time point no changes in the culture density were found (Fig. 1).

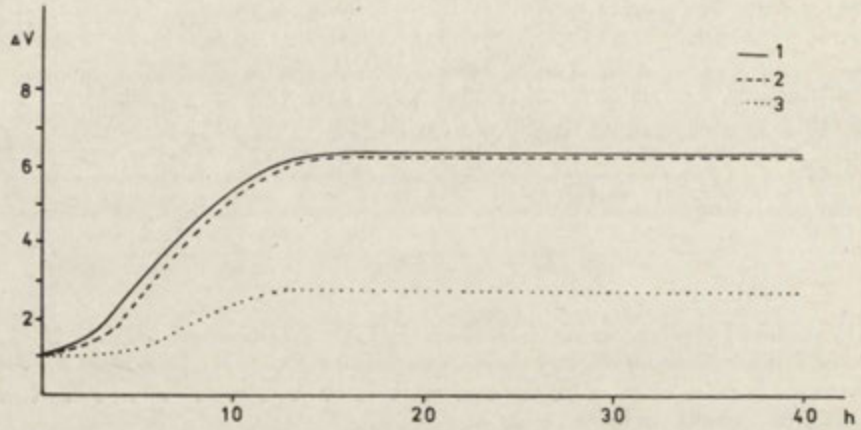


Fig. 1. Changes in the number of *Tetrahymena pyriformis* GL after washing and transferring the cells to an amino-acid-free-medium at various antibiotic concentrations in the culture. *The abcissa*: time (in hours) after washing the cells in an amino-acid-free-medium; *the ordinate*: an increase in the number of cells (dV). *1* — Control, *2* — Colistin 0.05 mM, *3* — Colistin 1 mM

The addition of the antibiotic to the culture in both concentrations at 12, 19, 24 and 31 h after washing the cells in the amino-acid-free-medium decreased the culture density to a non-significant degree as compared to the control (Szablewski — unpublished data).

The Effects of the Short-term Colistin Pretreatment on the Predivision Stomatogenesis and Oral Replacement

The cells derived from the late phase of exponential growth undergo the predivision stomatogenesis only. The transfer of such cells directly, i.e., without pretreatment with colistin or indirectly, i.e., after short-term pretreatment with colistin produced a gradual decrease in the number of cells undergoing PS and the emergence of cells undergoing OR, which gradually increased in number. The rate at which the two processes occurred is shown in Figs. 2 A, B and 3 A, B.

Irrespective of the duration of pretreatment with colistin and its concentration in the medium, the fraction of cells undergoing PS disappeared completely at 45 min after 0 time point. Subsequently the number of these cells increased to reach the maximum number at 2 h after 0 time point and then gradually fell down (Fig. 2 A, B, curves *1*, *2*, 3 and *4*). The differences in the number of cells undergoing PS in particular samples were not statistically significant.

The first cells undergoing oral replacement were observed 2 h after their transfer to the amino-acid-free-medium. Irrespective of the duration of the pretreatment and colistin concentration in the culture, the fraction of cells undergoing OR gradually increased during the initial 12 h of the experiment (Fig. 3 A, B; curves *1*, *2*, *3* and *4*). The number of cells undergoing OR did not differ significantly between the investigated samples and the control, with the exception of the culture in which the cells had been pretreated with colistin at a concentration of 1 mM for 30 min (Fig. 3 B, curve *4*).

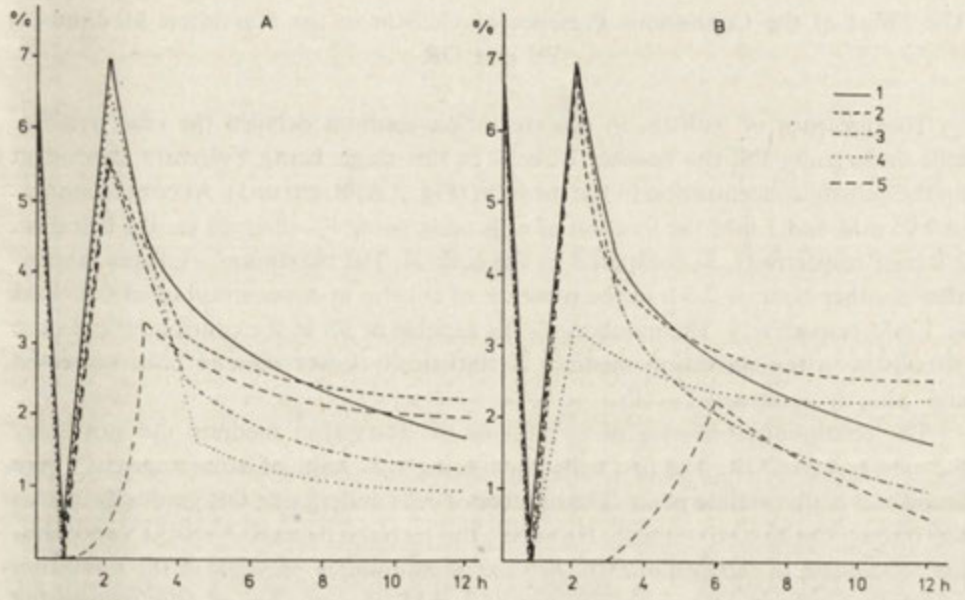


Fig. 2. Percentage of cells of *Tetrahymena pyriformis* GL undergoing predivision stomatogenesis according to the antibiotic concentrations in the medium, duration of cells' exposure to the antibiotic and duration of the experiment. *The abcissa*: time (in hours) after washing; *the ordinate*: percentage of cells undergoing predivision stomatogenesis. A — Colistin 0.05 mM, B — Colistin 1 mM, 1 — Control, *2* — Exposure to the antibiotic 5 min, *3* — Exposure to the antibiotic 15 min, *4* — Exposure to the antibiotic 30 min, *5* — Continuous presence of the antibiotic in the culture

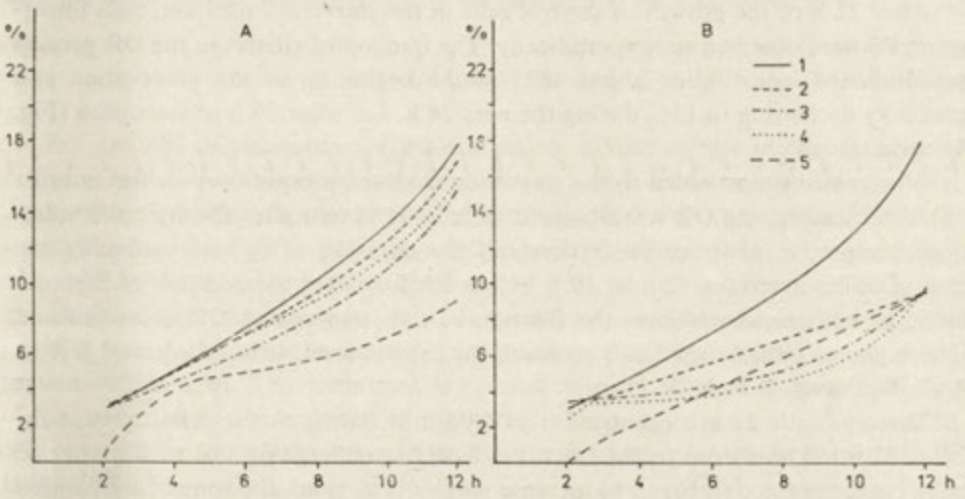


Fig. 3. Percentage of cells of *Tetrahymena pyriformis* GL undergoing oral replacement according to the antibiotic concentration in the medium, duration of cells' exposure to the antibiotic and duration of the experiment. *The abcissa*: time (in hours) after washing; *the ordinate*: percentage of cells undergoing oral replacement. A — Colistin 0.05 mM, B — Colistin 1 mM, *1* — Control, *2* — Exposure to the antibiotic 5 min, *3* — Exposure to the antibiotic 15 min, *4* — Exposure to the antibiotic 30 min, *5* — Continuous presence of the antibiotic in the culture

The Effect of the Continuous Presence of Colistin in the Starvation Medium on PS and OR

The presence of colistin in the starvation medium delayed the emergence of cells undergoing PS, the number of cells in this stage being evidently dependent on the colistin concentration in the medium (Fig. 2 A, B, curve *5*). At concentrations of 0.05 mM and 1 mM the fraction of cells undergoing PS emerged ca. 0.5 h and ca. 2 h later respectively, as compared to the control. The maximum level was reached after another hour or 2.5 h in the presence of colistin at concentrations of 0.05 mM or 1 mM respectively. The number of cells capable of PS in the continuous presence of colistin in the starvation medium is statistically lower than in both untreated and shortly pretreated cells.

The continuous presence of colistin in the starvation medium did not delay the emergence of OR. The first cells undergoing this kind of stomatogenesis were found at 2 h after 0 time point. The number of cells undergoing OR gradually increased throughout the experiment. However, the increase depended on the concentration of colistin in the medium. In the case of continuous presence in the starvation medium of colistin at a concentration of 1 mM, the number of cells undergoing OR was statistically lower as compared to the control (Fig. 3 B, curve *5*).

The Effect of Colistin upon the Number of Cells Undergoing OR According to the Duration of Starvation

After 22 h of the growth of control cells in the starvation medium, cells undergoing PS were observed only sporadically. The fraction of ciliates in the OR process predominated, constituting about 18% at the beginning of the observation and gradually decreasing to 13% during the next 24 h, i.e., after 36 h of starvation (Fig. 4, curve *1*).

When colistin was added to the starvation medium, a rapid drop in the number of ciliates undergoing OR was observed already at 45 min after the antibiotic addition, irrespective of its concentration and the duration of cell starvation. In the case of cells starved for 12 h or 19 h within 2 h following the addition of the antibiotic at both concentrations, the fraction of cells undergoing OR decreased and then began to increase gradually to reach the control level within the next 3 h (Fig. 4 A, B, curves *2* and *3*).

The antibiotic at a concentration of 0.05 mM added to the cells starvard for 24 or 31 h led to a drop in the fraction of ciliates undergoing OR to 0% after 45 min. Then, the fraction began to increase gradually to reach the control level within the next 3 h (Fig. 4 C, D, curve *2*). Colistin at a concentration of 1 mM added after 24 h starvation also led to a rapid decrease in the number of cells undergoing OR after 45 min, while after 1.5 h a new increase in the number of ciliates undergoing OR might be observed. The control level was also likely to be achieved in this case,

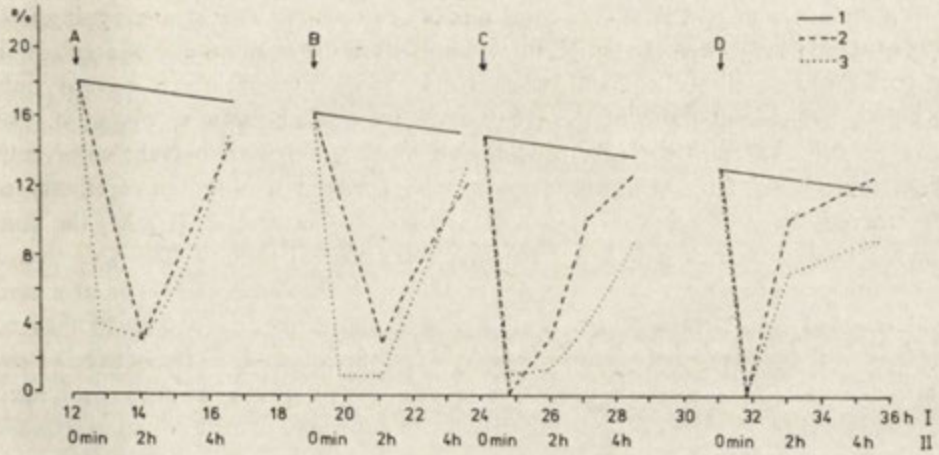


Fig. 4. Percentage of cells of *Tetrahymena pyriformis* GL undergoing oral replacement according to the antibiotic concentration in the culture, time of the antibiotic addition to the culture and duration of the experiment. *The abcissa*: time (in hours) after washing the cells in amino-acid-free-medium (I) and addition of the antibiotic (II); *the ordinate*: percentage of cells undergoing oral replacement. *1* — Control, *2* — Colistin 0.05 mM, *3* — Colistin 1 mM, A — Antibiotic added 12 h after washing, B — Antibiotic added 19 h after washing, C — Antibiotic added 24 h after washing, D — Antibiotic added 31 h after washing

though later than after 12 h or 19 h of cell starvation (Fig. 4 C, curve *3*). The same concentration of the antibiotic in the medium of cells starved for 31 h also produced a decrease in the number of ciliates undergoing OR to 0% after 45 min, followed by an increase in the number of such cells. However, a rapid growth in the number of cells undergoing OR lasted ca. 1.5 h, and then the increase was slight. In this case the control level was not likely to be achieved (Fig. 4 D, curve *3*).

Discussion

The analysis of the course of growth phases of the culture in the continuous presence in the starvation medium of colistin at both concentrations indicates that the starved *Tetrahymena* are capable of recovery. However, activation of the recovery mechanism is prolonged, since in the presence of colistin at a concentration of 1 mM in the medium the lag phase lasted 5 h, while the number of cells was first doubled after the next 4 h. The time necessary to double the number of cells, i.e., 4 h, was in this case the same as in the control culture and with the antibiotic present at 0.05 mM. The prolonged lag phase suggests an increased susceptibility of the starved cells as compared to the non-starved ones, since in the non-starved cells the addition of colistin at 1 mM produced a 2.5 h long lag phase (Szablewski 1984, 1985). The analysis of PS and OR kinetics leads to similar conclusion. Antibiotic addition always produced a decrease in the number of cells undergoing oral morphogenesis. However, the decrease was transient with the subsequent normal course of oral morphogenesis and the resumed kinetics of PS replacement by OR. The complete recovery, i.e., (1) normal course of oral morphogenesis, (2) kinetics

of PS replacement by OR, (3) the same number of cells capable of undergoing OR, was observed only when the antibiotic was present in the medium at a concentration of 0.05 mM. On the other hand, when 1 mM of the antibiotic was present, only the first and second parts of the recovery mentioned above were observed. The concentration of colistin in the medium was high enough to prevent some cells from undergoing OR. Accordingly, it may be assumed that the susceptibility of the starved cells was heightened, since at the same concentration (1 mM) the non-starved cells were capable of full recovery (Szablewski 1985). The fact that a lesser number of cells was able to undergo OR in the presence of colistin at a concentration of 1 mM in the control sample or with the antibiotic at 0.05 mM may be accounted for by the lengthened lag phase. After the addition to the culture of the antibiotic at a concentration of 0.05 mM, the lag phase was slightly prolonged as compared to the control and the cells in the starvation medium were capable of two divisions in both cultures. On the other hand, with colistin present at a concentration of 1 mM, the cells divided only once, although the time necessary to double the number of cells in this culture (after completion of the lag phase) was the same as in the remaining cultures.

A brief action of colistin prior to starvation did not alter the kinetics of PS replacement by OR, as compared to the control. A 30-min exposure of the cells to the antibiotic produced a decrease in the number of ciliates able to undergo PS, while the number of cells starting OR did not change. Parallel studies on PS in the case of cells kept in the nutrient medium demonstrated a similar nature of the changes observed (Szablewski et al. 1985). In the latter case, however, the correlation with the duration of *Tetrahymena* pretreatment with colistin and the antibiotic concentration in the medium was not that evident.

The findings are confirmed by other authors' observations. Satir (1971) demonstrated the recovery of *Tetrahymena* in the presence of actinomycin D, though the cells' response to the inhibitor of cell metabolism depended, among other things, on the cultural growth phase at which the inhibitor was added. Similar results were obtained using colistin, although the site and manner of actinomycin D and colistin action differ.

Another question is, after how long starvation period the cells are able to activate the mechanism of recovery. Is the capacity for initiating the mechanism responsible for recovery related to the duration of starvation, since Nilsson (1984) proved that in the cells kept in a starvation medium autophagy was active as early as after 30 of starvation? The present findings seem to indicate that cells starved for 12 h or 19 h, in spite of autophagy which occurs in them, are able to activate the mechanism of recovery. After 24 h long starvation the process of recovery in *Tetrahymena* in the presence of colistin at a concentration of 1 mM is already hindered and slower. On the other hand, after 31 h long starvation the cells are still capable of activating the process of recovery in the presence of colistin at a concentration of 0.05 mM, while at a concentration of 1 mM of the antibiotic the reactivation of the mechanism

of recovery is difficult and/or does not concern all the cells. It might be due to the fact that in the cells starved for 24 h or 31 h the processes of autophagy are so advanced that the ciliate's tolerance to the concentrations of the antibiotic employed in the study is dramatically reduced.

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The Site of Action of Colistin on *Tetrahymena pyriformis*

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Synopsis. The site at which the polypeptide antibiotic colistin was incorporated into the cells of *Tetrahymena pyriformis* GL was investigated, the observations being carried out after previous labelling of colistin with l-dimethylaminonaphtalene-5-sulphonyl chloride. Colistin was found to build into the cell membrane and the membrane of food vacuoles of the ciliates. Parallel studies employing fluorochrome demonstrated that the compound penetrated into the cells' cytoplasm.

Studies on *Procaryota* (Few and Schulman 1953, Newton 1953, 1954 a, b, Sebek 1967) and eucaryotic cells (phagocytes) (McKay and Kay 1964, Axline et al. 1967, Jawetz 1970) have demonstrated that the polymyxins, including colistin, do not penetrate into the cell. Through incorporation into the bacterial cell membrane (Kuryłowicz 1979) colistin alters its structure, simultaneously increasing its permeability (Sebek 1967, Russel 1977). Korzybski et al. (1977) suggest that colistin exerts a detergent-like action upon bacteria. Such an effect, however, has not been observed in the case of *Tetrahymena* (Szablewski 1984).

On the basis of earlier studies (Szablewski 1984, 1985) it has been established that *Tetrahymena* adapt to the permanent presence of non-lethal concentrations of colistin in the medium. The ciliates of this species respond alike to other drugs penetrating into the cell (Frankel 1965, Rasmussen and Zeuthen 1966, Roberts and Orias 1974). Kiersnowska (1984) is of an opinion that the ciliates' capacity for so-called physiological adaptation is characteristic of the species (genus) and does not depend on the site and mode of action exercised by a particular inhibitor.

The aim of the present study was to determine the exact site of action of colistin upon *Tetrahymena pyriformis* GL.

Material and Methods

The cells

The organism used in the study was an amicronucleate strain of the ciliate *Tetrahymena pyriformis* GL. The cells were cultivated in Erlenmayer flasks containing 25 ml of the medium (1.5% proteose-peptone + 0.1 yeast extract — Difco) at 28°C. Three hours after the inoculation (early exponential growth phase) either a complex antibiotic-fluorescent compound or fluorochrome was added to the cultures. The concentrations of both in the medium were 0.05 mM, 0.1 mM 0.5 mM, and 1 mM. The earlier studies (Szablewski 1984, 1985) proved 0.05 mM concentration of colistin to be the lowest dose effecting disturbances in the course of selected physiological functions in *Tetrahymena*, while 1 mM was the highest concentration of the antibiotic in the culture which did not kill cells in the investigated samples.

To exclude the fluorescence of the complex antibiotic-fluorescent compound or flurochrome absorbed on the cell surface, parallel observations were carried out on cells washed after 10 min out of the medium containing the labelled antibiotic or fluorochrome. The duration of the cells' exposure to the chemicals was determined on the basis of earlier studies (Szablewski et al. 1985), when it had been observed that even 5 min exposure of *Tetrahymena* to colistin produced morphogenetic and physiological changes in the cell. In the present study the cells were collected by centrifugation at ca. 700 rotations/min, for 5 min and washed twice with 1.5% proteose-peptone + 0.1% yeast extract or 0.9% NaCl or an amino-acid-free-medium (Frankel 1965).

The antibiotic used in the study was manufactured by the Polfa Pharmaceutical Company at Tarchomin. It is a mixture of colistin A (polymyxin E_1) and colistin B (polymyxin E_2). The product employed was colistin sulphate.

Preparation of the Complex Colistin-fluorescent Compound (ColDC)

A fluorescent derivative of colistin was prepared by coupling 1-dimethylaminonaphtalene-5-sulphonyl chloride (dansyl chloride) with the γ amino group of αγ-diaminobutyric acid radicals in the polymyxin molecule. Colistin was labelled according to the partially modified method originally suggested by Newton (1955). 400 mg colistin were dissolved in 5 ml 0.1 M sodium bicarbonate. 37.5 mg dansyl chloride (Sigma) were dissolved in 5 ml acetone. The dissolved dansyl chloride was added to the colistin solution. The mixture was stored for ca. 3 h at room temperature and then precipitated by adding approximately 200 ml methanol and stored at a temperature of +2°C for 24 h. Subsequently the mixture was centrifuged and washed three times with 99.6% ethanol. The precipitate was dried at a temperature of ca. 50°C and dissolved in 10 ml distilled water colistin concentration in the solution was approximately 5.25 mg/ml (3.75 mM).

Microscopical Observations

The samples were examined using a Carl Zeiss Amplival fluorescence microscope. An XBO 50 W lamp was employed as a light source. UG 1(1.5) + BG 12 were excitation filters and OG 4 was a barrier filter.

The photographs were taken on an NP-27 film (26 DIN) (Orwo) with the magnification of 200 ×.

Results

Following the addition of ColDC at all investigated concentrations to the *Tetrahymena* cultures, the fluorescence of the cell membrane, particularly pronounced in the cytostome, was found immediately upon addition of the labelled anti-

biotic, while the fluorescence of the food vacuole membrane was observed after 5–15 min, depending on the ColDC concentration in the medium (Pl. I 1). The effects were seen both in the cells washed out of the medium containing the labelled antibiotic and in the non-washed cells. At a concentration of 1 mM ColDC the cells died after about 15 min. Then fluorescence was observed inside the ciliates, caused by penetration of the complex into the cell cytoplasm. With the continuous presence of lower concentrations labelled colistin (0.05 mM, 0.1 mM and 0.5 mM) nearly all the cells survived beyond 48 h (the duration of the observation). However, at a concentration of 0.5 mM of the complex in the medium, 1 h after addition there was a larger percentage od dead cells (ca. 10%) than in the remaining samples. Moreover, 45 min after ColDC at a concentration of 0.5 mM was added to the culture, the motility of the cells decreased and the number of food vacuoles per cell was diminished (usually 1 or 2). On the other hand, at a concentration of 0.05 mM no such changes were observed as compared to the control. Analogous results were obtained in both cultures, those washed and those not washed out of the ColDC-containing medium.

The addition to the culture of dansyl chloride dissolved in acetone demonstrated its greater toxicity for the cells as compared to ColDC. Parallel studies of cell behaviour following the addition of acetone at appropriate concentrations did not reveal evident changes as compared to the control ciliates. At nearly all the concentrations of dansyl chloride employed, the compound was found to penetrate into the cells comparatively rapidly (Pl. I 2), thus leading to their death. On addition to the culture of dansyl chloride at a concentration of 1 mM (= 0.27 mg/ml), the cells died instantaneously. With dansyl chloride added to the culture at concentrations of 0.05 mM and 0.01 mM, the cell death occurred after 10 min and 30 min respectively. In order to produce fluorescence only of the cell membrane and food vacuole membrane, it was necessary to employ dansyl chloride concentrations lower than 0.05 mM (Szablewski — unpublished data). Also in that case, however, the cell motility and the number of food vacuoles per cell were lower than in the cells exposed to the same concentration of ColDC.

Discussion

The findings indicate that the site of action of colistin is the cell membrane of *Tetrahymena*. At all ColDC concentrations employed in living cells, fluorescence was observed exclusively in the cell membrane and the membrane of food vacuoles. Higher ColDC concentrations led to the instantaneous death of the cells and penetration of the complex into the cytoplasm, not infrequently with evident, transient densification around the macronucleus (Szablewski — unpublished data). A similar sensitivity of cells as that found to ColDC was observed in earlier studies (Szablewski 1984), which proved colistin concentrations in the medium

exceeding 1 mM lethal to *Tetrahymena*. The labelling of the cell membrane in living *Tetrahymena* and *Paramecium* was performed by Wyroba et al. (1981), employing a cycloheptamylose-dansyl chloride complex (CDC) at concentrations of 0.5 or 1.0 mg per ml. In the case of dead cells, however, CDC penetrated into the cells, producing a distinct fluorescence of the cytoplasm and other organelles.

The penetration of dansyl chloride into the cell and the absence of the effect at identical concentrations of ColDC indicate the cell membrane as the site of action of colistin.

The presence of ColDC in the membrane of food vacuoles may be accounted for by the process of phagocytosis, in the course of which the cell membrane provides material for the membrane of food vacuoles (Nilsson 1979). During studies conducted in the stage of *Tetrahymena* preadaptation to colistin (Szablewski — in preparation), a distinctly luminous spot was observed in the posterior part of the protozoon. In that case ColDC was probably incorporated into the cytopyge via the food vacuoles. Accordingly, colistin may be assumed to incorporate into the cell membrane of *Tetrahymena* without penetrating into its cell cytoplasm.

In view of these findings as compared to the results of earlier studies (Szablewski 1984, 1985) the suggestion put forward by Kiersnowska (1984) seems valid. According to it, the ciliates' capacity for physiological adaptation is a feature characteristic of the species (genus), independent of the site and mode of action exercised by a particular inhibitor of cell metabolism.

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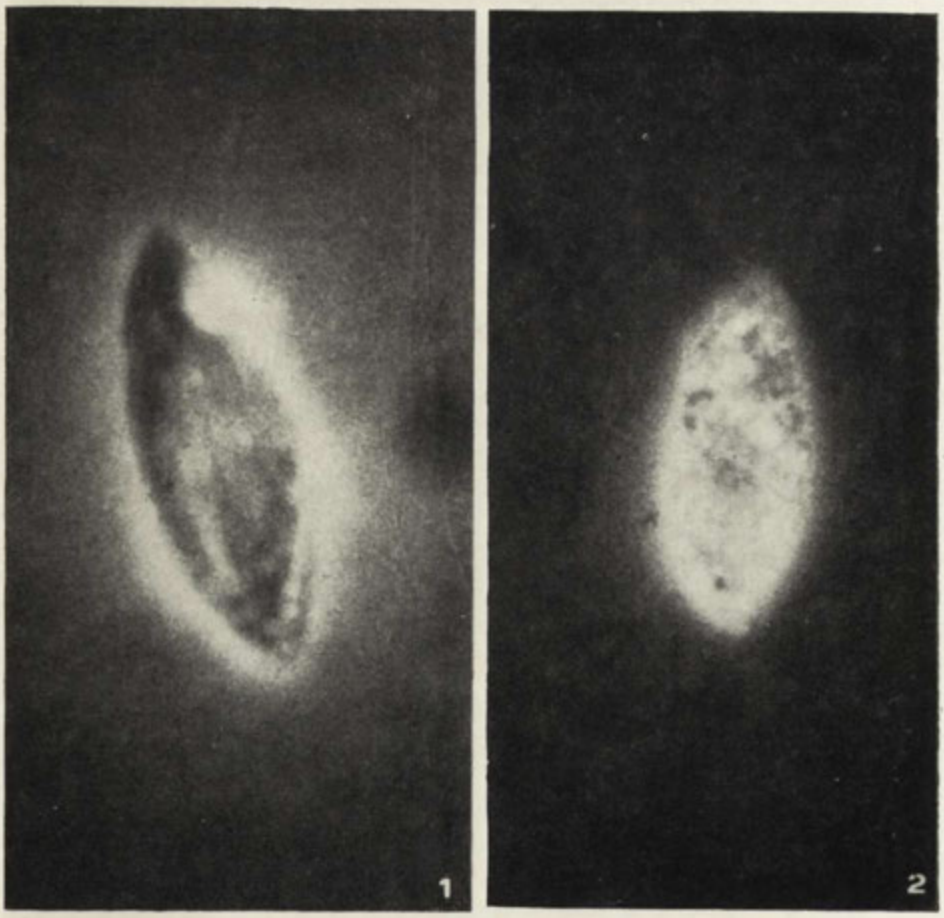
EXPLANATION OF PLATE I

1: *Tetrahymena pyriformis* GL after addition to the culture of Co1DC at a concentration of 0.5 mM. Visible fluorescence limited to the cell membrane, particularly pronounced in the cytostome. Living cells

2: *Tetrahymena pyriformis* GL after addition to the culture of dansyl chloride at a concentration of 0.5 mM. The dye penetrates into the cell cytoplasm, producing fluorescence of the entire *Tetrahymena*. Dead cells



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Clonal Life and Temperature Resistance in *Paramecium primaurelia*

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Synopsis. It has been demonstrated that in *Paramecium primaurelia* temperature resistance does not differ between the caryonides of the clone when cells are tested at the same fission and interfission ages (Crippa Franceschi et al. 1975). The present study shows that resistance varies in the clonal life course according to an oscillatory trend. Clones differing or not from each other in genic identity and/or in fission rate maintain such a trend with relative autonomy. So the temperature resistance, which varies inside the species according to the stock (Crippa Franceschi et al. 1971), is also seen to vary inside the stock according to the clone and inside a single genotype according to the fission rate.

The studies on temperature resistance had shown that the two caryonides of *Paramecium primaurelia* clone did not differ significantly in resistance, when tested at the same fission age since the autogamous process and at the same interfission age during a clonal life interval consisting of one hundred cell generations (Crippa Franceschi et al. 1975, 1977 a). Though unusually few differences occurred, they were not found at the subsequent tests, thus indicating that the different thermoresistances observed between the subclonal populations at a certain fission level did not mean a real and stable differentiation between the caryonides. Moreover, the series of the mean resistance values of the two caryonides at subsequent fission ages, suggested that the thermoresistance varied in the course of the clonal life examined. From analysis of variance it ensued that the differences in temperature resistance were related to the fission ages rather than to the caryonides (Crippa Franceschi et al. 1979). Furthermore, it appeared that, when the tests were performed at widely spaced fission ages, it was more difficult to point out some differences in thermoresistance, which were revealed more frequently when the fission interval between the tests was short. Hence, the character could be assumed to vary constantly during the clonal life course (Crippa Franceschi et al. 1977 b).

On account of these observations, the clone has been considered no longer by keeping the two caryonidal components apart, but as a whole, and the trend of temperature resistance has been analysed at close fission levels during a more or less extended interval of the exautogamous clonal life.

Material and Methods

The experiments were carried out on *Paramecium primaurelia* stock 90 cultivated at 25°C in a lettuce infusion buffered at pH = 6.8 and inoculated with *Klebsiella pneumoniae* one day before use. Sonneborn's methods (1950, 1970) of growing the cultures, of inducing and verifying the autogamy were followed.

Experimental Cultures

Autogamy was induced in starved lines after a series of daily reisolations. When 98% autogamous cells occurred in a sample consisting of over 100 individuals, a number of clones were isolated into depression slides containing 0.5 ml bacterized culture medium. The number of cells derived from a single one was noted daily and the $\log_2$ of this number represented the fission rate per day of that clone. The clones were selected on the basis of their daily fission rate; only the same fission age cells of a clone were daily reisolated in new depressions. The sum of fissions carried out from autogamy up to a given day was the fission age of the clone at that level.

Cytological tests were routinely performed in order to detect the macronuclear changes characterizing the autogamous cells. The dead sublines were replaced by sister sublines.

Some of the exautogamous clones were allowed to undergo the second autogamy in order to obtain genetically identical clones, i.e., homozygous clones for the same alleles. Both equal or different fission rate clones were selected. Therefore, three different groups were examined during a short (within 32 fissions) and an extended (up to 53 fissions) interval of the clonal life: genetically identical clones (M and Q) showing the same daily fission rate (3 fissions), genetically identical clones (B and C) displaying different daily fission rate (2 and 3 respectively) and genetically different clones (U, M and C) growing at the same daily fission rate (3 fissions).

Temperature Resistance Tests

Cells grown at the same fission rate were submitted to temperature resistance tests at increasing ages of the clonal life. At each level examined, synchronized cells (Franceschi 1957, 1958, Crippa Franceschi et al. 1975) were obtained by hand isolating in fresh culture medium the dividing cells at the same clonal age. Five hours after fission, most of the daughter cells were submitted to temperature resistance tests, and some others were allowed to grow in bacterized culture medium until their next division for the control of the cell cycle length. Therefore, each sample was quite homogeneous, consisting of cells at the same clonal and interfission ages. Temperature resistance tests were performed at 42.5°C by a thermostatic set equipped with a dissecting microscope. The cells of each sample were examined one by one until their death. The average of the death times in seconds of the cells of a sample at a given fission age represented the mean temperature resistance of the clone at that age.

Results

It has been observed that the temperature resistance of all the examined clones is not steady, but varies according to the fission age since autogamy. Such a varia-

tion appears to be more or less evident in relation to the examined clonal life interval. In fact, when the tests were carried out on not more than 32 exautogamous cell generations (Fig. 1), the temperature resistance showed a single peak at 20 fission level (clone C), preceded and followed by gradually increasing and de-

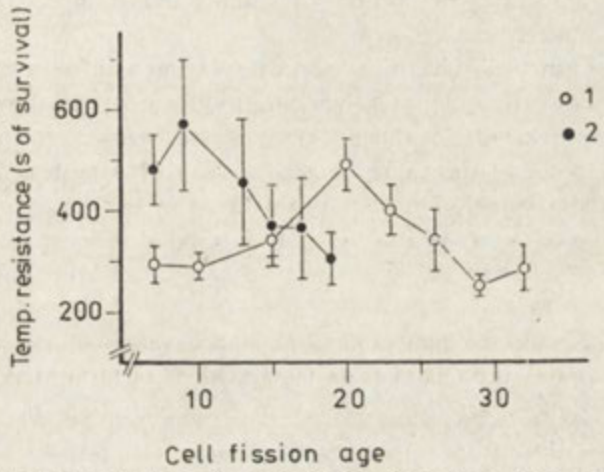

Fig. 1. Temperature resistance trend of genetically identical clones of *Paramecium primaurelia* displaying a different daily fission rate (2 fissions, clone B; 3 fissions, clone C). Mean temperature resistance in seconds of clone B (2) and clone C (1); vertical bars are 95% confidence limits; sample size = 30 cells

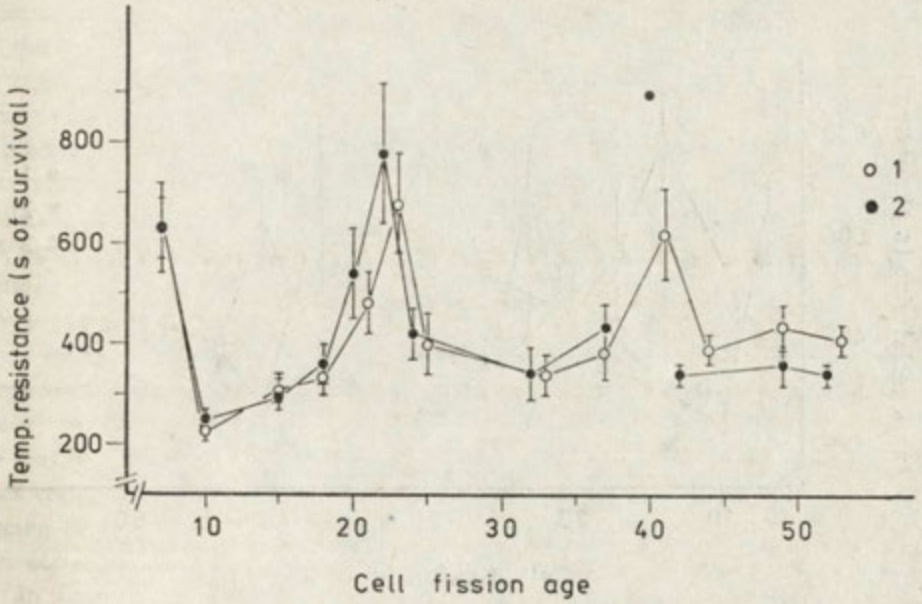

Fig. 2. Temperature resistance trend of genetically identical clones of *Paramecium primaurelia* showing the same daily fission rate (3 fissions). Mean temperature resistance in seconds of clone M (1) and clone Q (2); the value of 40 fission age is related to a sample consisting of a few cells; vertical bars are 95% confidence limits; sample size = 30 cells

creasing values respectively. On the contrary, when more extended intervals of the clonal life (up to 53 fissions) were considered, the temperature resistance displayed greater and lower values alternating rather regularly (Fig. 2).

If we consider genetically identical clones growing at the same fission rate (clones M, Q, Fig. 2), their temperature resistance trends appear similar and even overlapping in some tracts.

In the case of genetically identical clones displaying a different fission rate (clone B growing more slowly than clone C), their temperature resistance shows very inconsistent trends and more extended confidence limits related to slower growing clone (Fig. 1). Such an inconsistence of the values might be due to having tested the samples of different fission rate clones, 5 h after cell division in this case too; therefore, it might be that the cell cycle phases did not correspond. This result points out the value of the cell cycle and the importance of comparing synchronous samples in their interfission time.

While dealing with the temperature resistance values of genetically different clones growing at the same fission rate (clones M, C, U), the trends appear dissimilar, although the characteristic succession of the variations persists during the clonal life course (Fig. 3).

Referring finally to the trends of clones characterized by different genes and/or fission rate, a certain autonomy of the clone appears as regards the variations

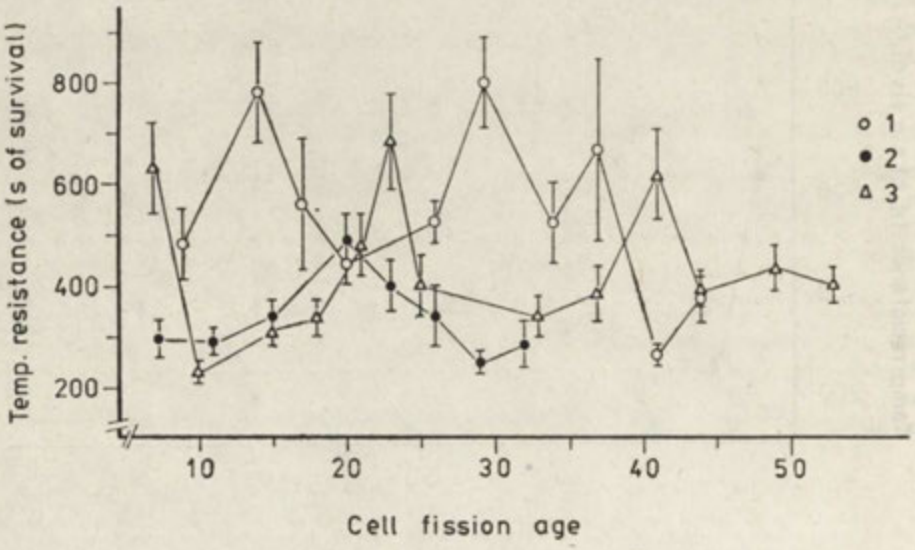

Fig. 3. Temperature resistance trend of genetically different clones of *Paramecium primaurelia* growing at the same daily fission rate (3 fissions). Mean temperature resistance in seconds of clone C (2), clone M (3) and clone U (1); vertical bars are 95% confidence limits; sample size = 30 cells

of temperature resistance: the clone tends to display its own range of values. This assumption is supported by the observation that genetically identical clones growing at the same fission rate show the same trend in the course of a common fission interval.

Discussion

From the above results, it ensues that the temperature resistance character shows a series of variations which are the more numerous the more the clonal life interval is extended. As fission age increases, the cells exhibit a resistance capacity which alternately decreases and rises according to an oscillatory pattern (unpublished data). Such a trend is pointed out by the frequency of the observations and by the kind of methodology employed for the resistance tests. In fact, the use of tests carried out at short fission intervals enabled us to note sudden variations in temperature resistance that could occur within few cell generations. Furthermore, the selection of synchronized cells allowed us to utilize quite homogeneous samples, both as regards clonal age and cell cycle, thus avoiding any mistake due to the variability in temperature resistance related to the metabolic activity changes occurring during the interfission time (Irlina 1969).

Quantitative variations correlated with clonal age have been pointed out in *Paramecium tetraurelia*: as clonal age progresses, there is a corresponding increase in the interfission time (Smith-Sonneborn and Klass 1974), in the sensitivity to caffeine and to ultraviolet light and X-rays (Smith-Sonneborn 1971, 1974, Fukushima 1974). On the other hand, when the clonal age increases, the cells exhibit a decrease in the macronuclear DNA content, in the DNA template activity and in the RNA synthesis (Klass and Smith-Sonneborn 1976), in endocytic capacity (Smith-Sonneborn and Rodermel 1976), and in giving increased life-span progenies at fertilization (Smith-Sonneborn et al. 1974). Many observations of the above-mentioned authors were carried out mainly with the aim of investigating cell ageing: therefore, the clones were not observed day by day, but each experiment took into consideration different clonal age clones, one arising from another. Other observations were applied to the same clone during its development: in this case, however, the tests were performed at widely spaced fission ages, so that it was impossible to reveal any oscillations of the character examined. Studies carried out on *Paramecium tetraurelia* at short fission intervals at until cell ageing of all examined clones, showed that the macronuclear DNA content oscillated according to the fission age (Schwartz and Meister 1975).

In the present study on *Paramecium primaurelia*, the proof of an oscillatory trend of temperature resistance clearly revealed during a clonal life interval consisting of 54 fissions, proposes new ways of research. One concerns the investigation of this character in older cells; another, the study of the relation between the va-

riations of temperature resistance capacity and some cell traits in the clonal life course. In this context, the maximum mean values of the macronuclear DNA content and the variation of the temperature resistance have been found immediately after autogamy; then, this independence of the two characters, observed in the early clonal life course, is followed by an inverse correlation between the macronuclear DNA amount and temperature resistance occurring during the subsequent interval of the clonal life examined. Therefore, the cell situation corresponding to a greater and a lower temperature resistance capacity is characterized by a lower and a higher macronuclear DNA content respectively (Delmonte Corrado et al. 1986).

Whatever the cell conditions related to the change of temperature resistance in *Paramecium primaurelia* may be, from our investigation it appears that such a character varies within the species according to the stock (Crippa Franceschi et al. 1971), within the stock according to the clone and the number of preceding autogamies (Ramoino et al. 1982) and depends, within a single genotype, on the fission rate.

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Biosynthesis of Prostaglandins in Pathogenic and Non-pathogenic Strains of *Acanthamoeba castellanii*

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Synopsis. The aim of the study was to examine the biosynthesis of prostaglandins in the pathogenic strain (309) and non-pathogenic strain (Neff) of *Acanthamoeba castellanii*. It was established that the activity of the synthetase of prostaglandins is almost identical in both strains and that the synthesis of endoperoxide prostaglandins is similar to that in order organisms up to the point when prostaglandin H is produced.

Prostaglandins, unsaturated fatty acids, are produced by all mammals, insects, crustaceans, molluscs and corals (Horrobin 1978) as well as parasitic worms (Grzywacz and Szkudliński 1984, Salafsky et al. 1984); they can be also found in plants (Cao and Cepero 1976). Until recently their presence in parasitic protozoa had been questioned (Gutteridge and Coombs 1977), however, they were discovered in *Entamoeba histolytica* (Das and Padma 1977) and in some strains of *Acanthamoeba* (Hadaś 1987).

The beginnings of biosynthesis of prostaglandins derivative of 20-carbonic unsaturated fatty acids can be traced back to 1964, when it was discovered that extracts of animal vesicular glands turned some of the acids into prostaglandins. Since then there has been a rapid progress in the studies of animal prostaglandins, though no investigation of biosynthesis of prostaglandins in parasitic protozoa and worms has been conducted.

The goal of the present study was to examine the biosynthesis of prostaglandins in pathogenic and non-pathogenic strains of *Acanthamoeba castellanii* and to investigate the influence of some co-factors and inhibitors on the process of oxygenation arachidonic acid.

Material and Methods

Strains of amoeba. The material was constituted of the trophozoites of *Acanthamoeba castellanii*. i.e., strain 309 — pathogenic for mice, isolated from cysts stored at 4°C since the time

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of the original isolation (Kasprzak and Mazur 1972), and strain Neff – non-pathogenic for mice, stored in a long-termed axenic culture since the original isolation (Neff 1957).

Amoebal culture. The amoebae were cultured axenically at 25°C in a liquid medium described by Červa (1966), composed of 2% Bacto-Casitone (Difco) and 10% horse serum.

Isolation of microsomes. The amoebae harvested from 4-day cultures were centrifuged at 900 g for 5 min and subsequently washed twice with physiological salt solution and once with PBS buffer pH 7.4. The sediment of protozoa thus obtained was suspended in PBS buffer. The cells were homogenized in teflon homogenizer at 4°C. The homogenates were centrifuged at 12000 g for 12 min. The sediment was disposed of, and the supernatant that contained microsomes was centrifuged at 100000 g for 60 min. The sediment of microsomes was suspended in 0.1 M Tris-HCl buffer, pH 8.2. The total protein content in the suspension was determined by the method of Lowry et al. (1951).

Oxygraphic measurements. Those measurements were conducted using the oxygen electrode (Yellow Springs Instrument Co.). The incubation was performed in containers filled with 0.9 ml Tris-HCl buffer (100 mM pH 8.2) and 0.1 ml of microsomal preparation with the strength of 1 to 5 mg of protein to ml, assuming as 100% the saturation of the solution with oxygen. The consumption of oxygen by microsomes was measured at the moment when arachidonic acid was added. Endogenic consumption of oxygen was measured in specimens without substrate. To eliminate the endogenic consumption of oxygen by microsomal specimens that could contain other oxidases, p-chloromercuribenzoic acid (pCMB) with the final concentration of 0.013 mM or potassium cyanide (KCN) with the final concentration of 1.2 mM were added.

Table 1

The coefficient 50 per cent of inhibition (IC_{50}) of the synthetase of prostaglandins

Inhibitors	IC_{50}* (μM/l)	
	Strain 309	Strain Neff
acetylosalicylic acid	195	215
phenylobutazone	151	140
indomethacin	0.2	0.3
hydroquinone	> 1000	> 1000
aminophenazone	> 1000	> 1000
tryptophan	> 1000	> 1000

* The values of IC_{50} are calculated from the curve of regression of of the impediment of formation of endoperoxide protaglandins at 100 μM concentration of arachidonic acid.

Stimulatory and inhibitory effects. The influence of the activators and inhibitors of cyclooxygenization (Table 1) was examined by adding them to the incubation medium in different concentrations. The coefficient 50 per cent of inhibition (IC_{50}) was calculated from the curve of regression of the impediment of formation of the endoperoxide prostaglandins by the examined compounds.

Results

The consumption of oxygen by the microsomes in the process of biosynthesis of prostaglandins in the non-pathogenic strain (Neff) was 17.2 (±3.2) nAtoms 0_2 /min/mg of protein, and 19.3 (±2.8) nAtoms 0_2/min/mg of protein in the patho-

genic strain (309). The endogenic consumption of oxygen by the synthetases of prostaglandins in both strains was 0 to 1 nAtoms O_2/min/mg of protein.

The optimum concentration of arachidonic acid in the oxygraphic studies was 100 to 200 μM. The spectrum of pH activity of the synthetases was pretty broad and was contained between 7.0 to 8.8 pH.

The coefficient 50 per cent of inhibition (IC_{50}) of the synthetase of prostaglandins by the examined compounds is shown in Table 1.

The first three compounds belong to the category of typical inhibitors of synthesis of prostaglandins, the other three are included among the so-called "sweepers" of free radicals or co-factors of peroxidation of the converison of prostaglandin G into prostaglandin H. At low concentration of the arachidonic acid (100 μM), the "sweepers" of free radicals act as strong inhibitors of the oxygen consumption by the synthetase of prostaglandins. With the same concentration of the compounds but high concentration of arachidonic acid (300 to 1000 μM), these compounds act as activators of the enzyme. Added to the incubation container after arachidonic acid had been added, the "sweepers" of free radicals at the concentration of 50 to 100 μM activated the synthesis of prostaglandins by 10 to 20 per cent.

Discussion

The majority of hitherto existing studies of prostaglandins of parasitic protozoa were concerned with detecting their presence in the cells and with their assumed activity. The synthesis of prostaglandins in not yet quite clear. From the beginning it was clear that particular several-stage chemical conversions of fatty acids could not be catalyzed by a single enzyme. Particular stages of the synthesis of prostaglandins were presented in the study by Robak and Kasperczyk (1979) and the monographic work by Zaorska (1986). To recapitulate, it can be said that the synthesis of prostaglandins depends on arachidonic acid produced, and the acid appears in esterified lipids of various types. The cycle of transformation begins with phospholipase A_2, the enzyme whose presence in the lysosomes of many organisms was established, and among others in protozoa (Visvesvara and Balamuth 1975, Misra et al. 1983). The synthesis of prostaglandins begins with the enzyme known as the synthetase of endoperoxides of prostaglandins (E.C. 1.14.99.1) and that very enzyme was the subject of the present study. The enzyme can catalyze three stages of reaction: (1) lipoxygenation, which produces 11-hydroperoxyeikozatetraenic acid (11-HPETE), (2) cyclooxygenation, which produces prostaglandin G, (3) peroxidation, which effects in the production of prostaglandin H. The characteristic feature of reactions catalyzed by the synthetase of prostaglandins is a slower pace of reactions. The slowering is most probably due to the emergence of free hydroxyl radicals that damage the protein of the enzyme. The role of the "sweepers" of free radicals is to eliminate these agents. The inhibiting activity of such "swee-

pers" as tryptophan, aminophenazone and hydroquinone, established during the present investigation, confirms earlier presumptions concerning the necessity of vestigial quantities of free radicals to initicate the reaction of the oxygenation of arachidonic acid. The vestigial quantities of free radicals can be eliminated by minimal quantities of 50 to 100 μM of the above-mentioned compounds. The adding of the "sweepers" before the oxygenation of arachidonic acid is begun, often effects in complete stoppage of the synthesis of prostaglandins; on the other hand, if they are added after the reaction has begun, the effect is activation and lack of symptoms of inactivation of the enzyme.

The above-mentioned investigations lead us to a conclusion that the synthetase of the endoperoxides of prostaglandin is characterized by features similar to those of the enzymes obtained from other tissues of animals. It possesses similar or slightly lower susceptibility to typical inhibitors of oxygenation. So far it is difficult to establish why the quantity of prostaglandin F_{2a} is higher in the pathogenic strains of *Acanthamoeba* (Hadaś 1987). The activity of the synthetases of prostaglandins in both studied strains is pretty similar. It should be said that the process of the synthesis of prostaglandin, up to the stage of the creation of prostaglandin H, is identical both in the pathogenic and non-pathogenic strains as well as in other organisms (Fig. 1). The differences in creating other prostaglandins can be observed only after prostaglandin H has been produced. This prostaglandin H is undurable

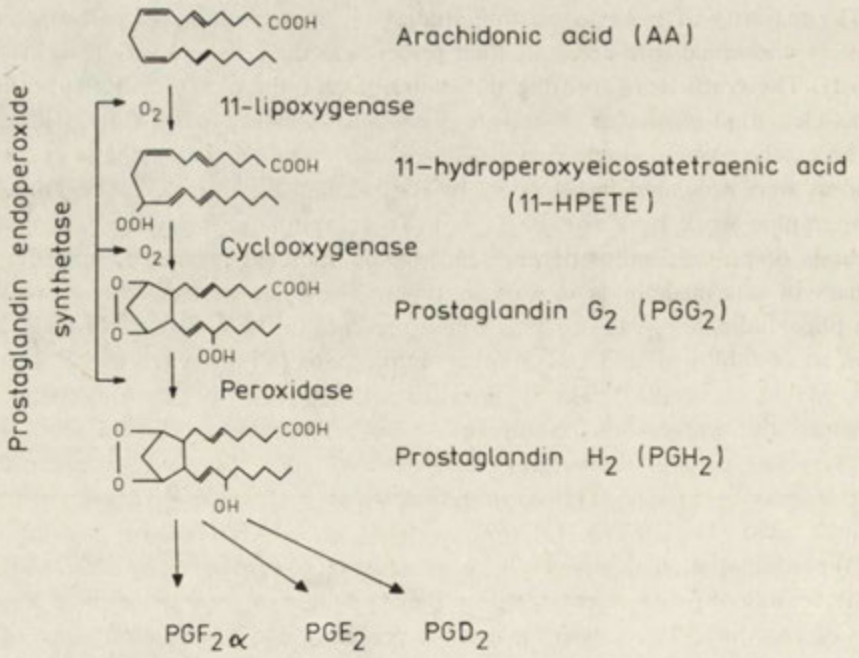

Fig. 1. The course of the biosynthesis of prostaglandins from arachidonic acid in amoebae and other animals

and becomes easily transformed, both enzymatically and non-enzymatically, to more durable prostaglandins. Investigations concerning the possiblity of controlling the synthesis of these compounds *in vitro* and *in vivo* should be continued, as they may eventually help to explain the differences in the composition of prostaglandins of both pathogenic and non-pathogenic strains and, in this way, possibly to throw some light on the mechanisms of pathogenicity.

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Leidyana guttiventrisa sp. n. A New Cephaline Gregarine (*Apicomplexa*: *Eugregarinida*) Parasite of a Gryllid of West Bengal, India

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Synopsis. A new cephaline gregarine *Leidyana guttiventrisa* sp. n. (*Apicomplexa*: *Eugregarinida*) has been described from the midgut of a gryllid *Plebeiogryllus guttiventris* (Walker) from West Bengal, India. The characteristic features of the gregarine are: epimerite lance-shaped, sporadin solitary — 206.5 μm long, gametocyst egg-shaped with unequal gametocytes, sporulation through three short sporoducts, barrel-shaped sporocyst — 8.8 μm × 6.4 μm in dimension, LP:TL = 1:4.68 and WP:WD = 1:1.2. The present gregarine has been compared with all the *Leidyana* spp. reported from the gryllid hosts to establish its distinctiveness.

During the course of investigation about the cephaline gregarine of arthropods, a gregarine species has been recovered from the midgut of a gryllid insect *Plebeiogryllus guttiventris* (Walker) from West Bengal, India. It has been described here as *Leidyana guttiventrisa* sp. n. for several of its characteristic features disscused latter.

Material and Methods

The hosts insects were collected from the field under the stones during the rainy season (June to September) and were brought alive to the laboratory where their alimentary canals were dissected out with 0.5% saline water and were examined for the gregarine parasite under the dissecting microscope. The smears of the infected midgut content were made on clean glass slides and were fixed in Schaudin's fluid and subsequently were stained with iron alum haematoxylin method. The heavily infected midguts were fixed in alcoholic Bouin's fluid, 8 μm thick sections were cut and were stained with iron alum haematoxylin. The gametocysts, collected from the midgut lumen of the host, were placed in the moist chamber in order to observe the subsequent development. The sporocysts were treated with Lugol's iodine solution and were examined under the oil immersion lens of the microscope (Olympus). The diagrams were made with the aid of a camera lucida and the measurements were given in micrometer (μm).

Observations

Fifteen percent of the insects *Plebeiogryllus guttiventris* (Walker) were found to be parasitized by the gregarine.

Trophozoite. The trophozoites were not found freely in the lumen of the host. However, they were found hanging from the midgut epithelium when the sections of the infected midgut of the host were examined. Each trophozoite was three-segmented, consisting of conical to lance-shaped epimerite, a rectangular protomerite and an elongated deutomerite with rounded end. The nucleus in the deutomerite was ovoidal in shape. The largest trophozoite was 42.0 μm × 11.0 μm in dimension (Fig. 1 *1*).

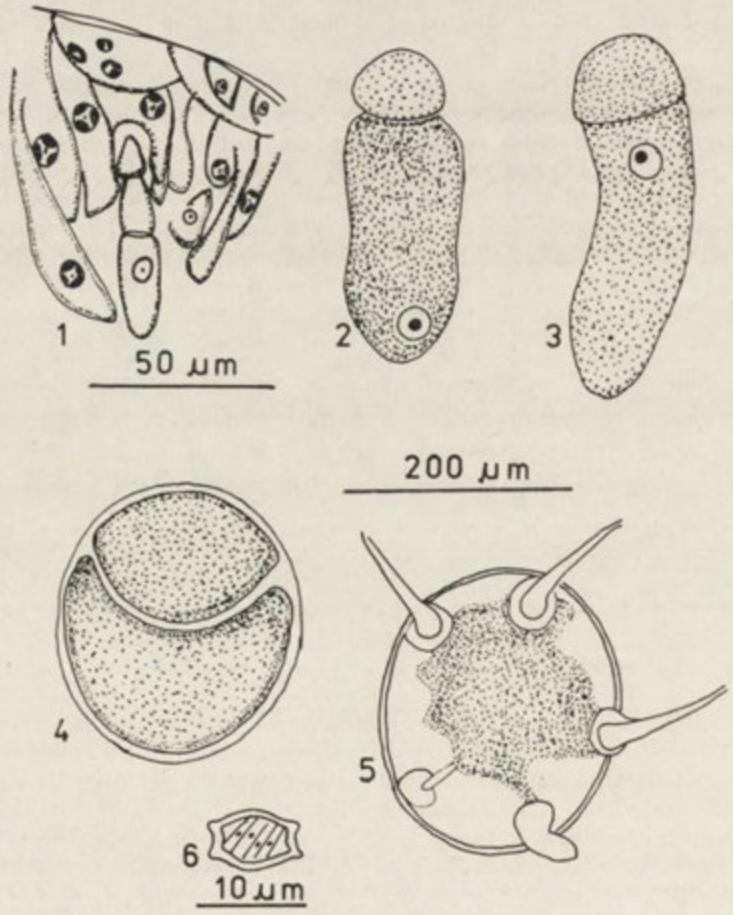

Fig. 1 *1–6*. Various stages in the life history of *Leidyana guttiventrisa* sp. n. *1* — A trophozoite hanging from the midgut epithelium of the host. (T.S. of the midgut of the host), *2–3* — Sporadins in various forms, *4* — An early gametocyst with unequal gametocytes, *5* — A mature gametocyst with sporoducts, *6* — A barrel-shaped sporocyst with sporozoites

Sporadin. The sporadins were freely found in the lumen of the midgut of the host. There were two-segmented structures consisting of dome-shaped (Fig. 1 *2*) to hemispherical (Fig. 1 *3*) protomerite and almost cylindrical deutomerite with rounded end. The nucleus was spherical and situated posteriorly in the deutomerite. The largest sporadin was 206.5 μm × 73.5 μm in dimension.

Gametocyst. The gametocyst obtained from the midgut lumen of the host was oval to egg-shaped with two unequal gametocytes visible at the early stage of development (Fig. 1 *4*). The ectocyst around the gametocyst was absent. After 48 h of development in the moist chamber the gametocyst gave rise to three short sporoducts through which the barrel-shaped sporocysts were released in chain or singly (Fig. 1 *5*). The dimension of the gametocyst was 231.0 μm × 196.0 μm.

Sporocyst. The sporocysts were barrel-shaped and measured 8.8 μm × 6.4 μm in dimension (Fig. 1 *6*) with inconspicuously visible sporozoites in them.

Measurements (in μm). The measurements of 2 trophozoites and 11 sporadins are given in Table 1.

Discussion

As the gregarine possesses a solitary sporadin, dehiscence of gametocyst through sporoducts and barrel-shaped sporocysts, it is assigned to the genus *Leidyana* Watson, 1915. So far only 8 species of cephaline gregarines have been reported from the midgut of the gryllid insects (Watson 1915, 1916, Corbel 1967, Geus 1969, Hoshide 1973, 1978, Haldar and Sarkar 1979 and Hoogar and Amoji 1986) (see Table 2). Regarding the shape of the trophozoites, sporadins and sporocysts, the present gregarine resembles superficially with *Leidyana erratica* (Crawley, 1903) Watson, 1915, *L. suzumushi* Hoshide, 1973, *L. oviformis* Hoshide, 1978, *L. linguata* Haldar and Sarkar, 1979 and *L. bimaculata* Hoogar and Amoji, 1986. Moreover, in having ovoidal to egg-shaped gametocyst, it also resembles *Leidyana gryllorum* (Cuenot, 1887) Watson, 1916. However, the present gregarine differs from all the above mentioned *Leidyana* species in having conical to lance-shaped epimerite, ovoidal to egg-shaped gametocyst with unequal gametocytes, lesser number of short sporoducts and larger sporocyst. It is also described from a new gryllid host. In view of such differences, I propose that the gregarine should be considered a new species and the name *Leidyana guttiventrisa* sp. n. be given to it.

Material. Holotype on slide No. 0_3L-8, prepared from the midgut content of *Plebeiogryllus guttiventris* (Walker) collected from Chinsurah by N. K. Sarkar on 18 July 1986. Paratypes on other slides are the same as holotype.

ACKNOWLEDGEMENT

Author expresses sincere thanks to Prof. A. Pramanik, Head of the Dept. of Zoology, R. B. C. College, Naihati for laboratory facilities.

Table 1

The detail measurements of two trophozoites and eleven sporadins are given below (μm)

Sl. No.	TL	LE	WE	LP	WP	LD	WD	DN	LP : TL	WP : WD
(1)	40.0	8.75	5.0	11.25	7.5	20.0	—	—	—	—
(2)	42.0	7.50	6.0	12.50	7.5	22.0	11.0	—	—	—
(3)	206.5	—	—	49.00	77.0	157.5	73.5	14.8	1 : 4.2	1 : 0.95
(4)	161.0	—	—	35.00	45.5	126.0	38.5	16.0	1 : 4.6	1 : 0.85
(5)	136.5	—	—	28.00	40.25	108.5	28.0	17.0	1 : 4.9	1 : 0.69
(6)	147.0	—	—	35.00	42.00	112.0	50.75	14.7	1 : 4.2	1 : 1.21
(7)	148.75	—	—	29.75	38.5	119.0	38.5	17.5	1 : 5.0	1 : 1.0
(8)	176.75	—	—	36.75	56.0	140.0	57.75	17.5	1 : 4.8	1 : 1.03
(9)	122.5	—	—	24.50	31.5	98.0	42.0	17.5	1 : 5.0	1 : 1.33
(10)	138.25	—	—	33.25	31.5	105.0	42.0	14.0	1 : 4.2	1 ; 1.33
(11)	105.0	—	—	21.00	35.0	84.0	52.5	17.5	1 : 5.0	1 : 1.5
(12)	98.0	—	—	21.00	38.5	77.0	49.0	16.45	1 : 4.7	1 : 1.27
(13)	171.5	—	—	35.00	61.25	136.5	73.5	21.0	1 : 4.9	1 : 1.2

LP : TL = 1 : 4.2–5.0 (4.68); WP : WD = 1 : 0.69–1.27 (1.12).

Abbreviations: TL – Total length; LE – Length of epimerite; WE – Width of epimerite; LP – Length of protomerite; WP – Width of protomerite; LD – Length of deutomerite; WD – Width of deutomerite; DN – Diameter of nucleus; ( ) = Mean; LP : TL = Ratio of the length of protomerite to total length; WP : WD – Ratio of the width of protomerite to the width of deutomerite.

Table 2

The comparison of the *Leidyana* spp. reported from the gryllid hosts

Parasite (Host)	Epimerite	Sporadin (Max. TL) (LP : TL) (WP : WD)	Gametocyst	Sporocyst	Locality
1	2	3	4	5	6
Leidyana erratica (Crawley, 1903) Watson, 1915 *Gryllus abreviatus, G. pensilvanicus*	Spherical knob	500 μm 1 : 5–7 1 : 1.3–1.7	Spherical with ectocyst, 350 μm in diam. 1–12 sporoducts	Barrel shape 6 μm × 3 μm	North America
L. gryllorum (Cuenot, 1897) Watson, 1916 (*Acheta domesticus, Gryllus assimilis, G. campestris*)	–	420 μm 1 : 5 1 : 1.1	Spherical or oval, 190–240 μm, 3–8 sporoducts	Barrel shape, 7 μm	France
L. saigonensis Corbel, 1967 (*Gryllus bimaculatus, Gryllodes sigillatus*)	–	350 μm	–	Dolioform 7.5 μm × 3.5 μm	Vietnum
L. oblongata (Dufour 1837) Geus, 1969 (*Gryllus campestris, Nemobius silvestris*)	Spherical knob 18–24 μm	210 μm 1 : 4.8–8.6	–	–	France
L. suzumushi Hoshide, 1973 (*Homoeogryllus japonicus*)	Sessile knob	348 μm 1 : 6.1 1 : 1.4	Spherical, 200 μm diam., 4–6 sporoducts	Barrel shape, 3 μm × 5.5 μm	Japan
L. oviformis Hoshide, 1978 (*Pteronemobius fascipes, P. taprobanensis*)	Spatula shape, 3 μm × 18 μm	350 μm 1 : 5.6 1 : 1.7	Spherical 155 μm in diam.	Barrel shape, 6 μm × 3 μm	Japan

Table 2 cont.

1	2	3	4	5	6
L. linguata Haldar and Sarkar, 1979 (*Pteronemobius concolor*)	Tongue like 15.7 μm × 7 μm	415.8 μm 1 : 5.5 1 : 1.3	Spherical 75 μm–154 μm in diam.	Cylindrical 9.8 μm × 4.1 μm	India
L. bimaculata Hoogar and Amoji, 1986 (*Gryllus bimaculatus*)	Spherical to papilla like, 3–7 μm × 2.5 μm	315 μm 1 : 3–7 1 : 1–2	Oval, 330 μm × 350 μm 10 sporoducts	Dolioform 3.5 μm × 5 μm	India
L. guttiventrisa sp. n. (*Plebeiogryllus guttiventris*)	Conical to lance shape 8.7 μm × 7.5 μm	206.5 μm 1 : 4.2–5 1 : 0.69–1.12	Ovoid to egg shape, 231 μm × 196 μm, 3 sporoducts	Barrel shape 8.8 μm × 6.4 μm	India

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Observations on Cephaline Gregarine *Steinina lunata* sp. n. (*Actinocephalidae*) from *Myllocercus undecimpustulatus maculosus*

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Synopsis. Steinina lunata sp. n. has been reported from the intestine of the cotton grey weevil, *Myllocerus undecimpustulatus maculosus* Desbrocher. It is described in details with special reference to its morphology, life-history stages and taxonomic position.

Léger and Duboscq (1904) for the first time created the genus *Steinina* for the inclusion of *Steinina ovalis* which they encountered in the intestine of the *Tribolium molitor*. After the establishment of the genus *Steinina* important contribution to this genus have come from Ishii (1914), Watson (1955). Recently Sarkar and Chakravarty (1969) added a few more species under this genus. The chronological list of *Steinina* spp. described so far from coleopterans and their localities is given in Table 2 (see Systematic Position).

The present communication records one more new species of the genus *Steinina* Léger et Duboscq from the adults of *Myllocerus undecimpustulatus maculosus* (*Insecta*: *Coleoptera*) collected at various places in Punjab, India. Observations on their morphology, life history, affinities, and seasonal intensity are noted and particulars about the holotype and paratype materials are also described.

Material and Methods

The adults and nymphal stages of this insect were collected from cotton fields, *Zizypus* sp. (Beri) and were fed with same type of food. The digestive tract of freshly killed insects were cut open in 0.75% saline solution. The semidried smears were immersed in Schaudinn's fixative and stained with Heidenhain's iron haematoxylin.

The ratios used in this paper are those of length of protomerite to total length (LP : TL), width of protomerite to width of deutomerite (WP : WD) and width of protomerite to length of protomerite (WP : LP). The following abbreviations are used in this paper: LE – length of epimerite, LP – length of protomerite, LD – length of deutomerite, TL – total length, WE – width of epimerite, WP – width of protomerite, WD – width of deutomerite.

Observations

Morphology

Development. The earliest stage in the epithelium of the host's midgut, as revealed in sections, is a rounded body (2.7 μm) (Pl. I 1). The nucleus is spherical and has a peripherally placed endosome. While it grows, it may involve more than one adjoining cell. Later, the body of the parasite is partitioned by a septum into an anterior protomerite (4.5×8.1 μm) and posterior deutomerite (6.3×8.1 μm) (Pl. I 2, 3). As it grows further, an epimerite develops at the tip as a elongated process. Then it attains the maximum size and the deutomerite become almost rounded and has a 19.6 μm diameter (Pl. I 4).

The Cephalont are found in small numbers, solitarily and in various sizes. They appear darker in transmitted light as the cytoplasm is highly granular. The epimerite is a elongated motile digitiform process changing into a dark coloured half moon-shaped structure. The epimerite measures 3.6×2.7 μm. The protomerite is roughly triangular with angled lateral sides (3.6×6.3 μm) and the deutomerite is roughly globular and measures 10.8 μm in diameter. It has an anteriorly placed rounded nucleus (3.6 μm) see Table 1.

The Sporont (Pl. I 5, 6) are also found in lesser number, solitarily and in various sizes. The cytoplasm is highly granular and dense. The epimerite is dark coloured, half moon-shaped with a concave base lying on the protomerite (4.5×8.1 μm). The less granulated protomerite is roughly pyramidal-shaped in the young specimen and twice as wide as its length. A septum is present in the middle or nearer the deutomerite in the protomerite. The deutomerite is separated from the protomerite by a thick septum and a distinct constriction. Deutomerite is a globular structure enclosing a big nucleus. The nucleus measures 6.3 μm and is situated in the middle of the deutomerite. In the younger individuals it is proportionally larger in relation to the size of the gregarine. It is spherical with a prominent nucleolus and reticulated nucleoplasm.

In the young sporont, the ratio between LP:TL lies between 1:2.6–3.4 and width of deutomerite is more than that of protomerite: 1:1.3–1.75.

Cysts (Pl. I 7) measures 74 μm. The cyst wall is thick. Immature cyst with gametes are also observed. Spores are unknown.

Material. Holotype, trophozoites on slide No. MUM-1a and 1/b prepared from the contents of midgut and hindgut of the weevils, collected from Patiala. Deposited at the Zoology Dept., Punjabi University, Patiala.

Paratypes. Many specimens (trophozoites, sporonts, and gametocystes) on the same slide as the holotype. Other particulars as for holotype.

Seasonal intensity and site of infestation. The intestine of the host was found heavilly infected with this gregarine during the rainy season i.e., from late April to August. On an average 25% of the hosts are found infected during the rainy season.

Table 1

Measurements of cephalonts and sporonts of *Steinina lunata* sp.n.

Cephalont	
Length of epimerite LE	— 3.6 μm
Width of epimerite WE	— 2.7 μm
Length of protomerite LP	— 3.6 μm
Width of protomerite WP	— 6.3 μm
Length of deutomerites LD	—10.8 μm
Width of deutomerites WD	—10.8 μm
Total length	—18 μm
Diameter of the nucleus	— 3.6 μm
Sporont	
Length of epimerite LE	— 4.5 μm
Width of epimerite WE (half moon-shaped)	— 8.1 μm
Length of protomerite LP	— 5.4 μm
Width of protomerite WP	— 7.92 μm
Length of deutomerite LD	—10.26 μm
Width of deutomerite WD	—10.34 μm
Diameter of the nucleus	— 5.4 μm
Ratios: LP:TL 1:2.88	
WP:WD 1:4.76	
WP:LP 1:0.686	

Systematic Position

This new gregarine belongs to the family *Actinocephalidae* Léger, 1892, and the genus *Steinina* Léger et Duboscq, 1904, by having solitary sporonts, complex epimerite, and dehiscence of cyst by simple rupture.

The new species is compared with *Steinina ovalis* Léger et Duboscq, *S. obconica* Ishii, *S. rotunda* Watson, *S. harpali* Watson, *S. microgonusae* and *S. alphitobiusae* Sarkar et Chakravarty. The new species differs from all the known species of the genus *Steinina* by having unique half moon-shaped epimerite. However, it resembles superficially with *Steinina microgonusae* known from the intestine of *Anoplogenius microgonus* in possessing the same position of the epimerite but differs on other basis (Tables 2, 3).

The above mentioned differences clearly show that this gregarine does not agree with any known species of the genus. Thus it is certainly an unnamed species and has been described here as a new species in detail. The specific name pertains to the unique shape of the epimerite.

Table 2

Chronological list of *Steinina* species described from coleopterans and their localities

Steinina sp.	Host (S)	Locality
Steinina ovalis (Stein) Léger and Duboscq, 1904	*Tenebrio molitor*	France
S. obconica Ishii, 1914	*Tribolium ferrugineum*	Province of Izu and Japan
S. rotunda Watson, 1915	*Amara augustata*	St. Joseph
S. harpali Watson, 1915	*Harpalus pennsylvanicus longior*	Urbana
S. alphitobiusae Sarkar and Chakravarty, 1969	*Alphitobius piceus*	India
S. microgonusae Sarkar and Chakravarty, 1969	*Anoplogenius microgonus*	India

Table 3

Comparison between *Steinina microgonusae* Sarkar et Chakravarty and *Steinina lunata* sp. n.

	Steinina lunata sp. n.	*Steinina microgonusae*
Epimerite	Half moon-shaped	Saucer-shaped
Protomerite	Pyramidal-shaped, a septum present in the middle or more nearer to the deutomerite	Dome-shaped
Deutomerite	Globular structure	Broader near the septum and gradually tapers towards the posterior end
Nucleus	Spherical with a prominent nucleolus and reticulated nucleoplasm	15 to 25 small chromatin granules, nucleoplasm is homogeneous
Sizes	LP 5.4 μm	30–35 μm
	WP 7.98 μm	50–60 μm
	LD 10.26 μm	50–90 μm
	WD 11.34 μm	50–60 μm
	TL 15.66 μm	90–140 μm
Ratios	LP : TL 1:2.88	1:3.6
	WP : WD 1:1.476	1:1
Cysts	74 μm	unknown
Spores	unknown	unknown

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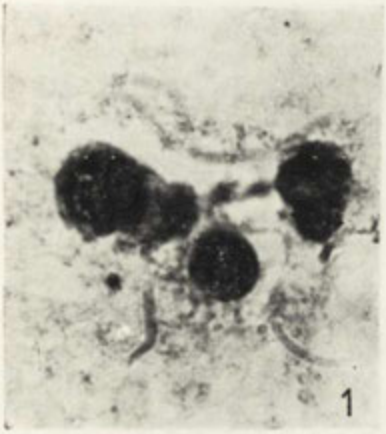
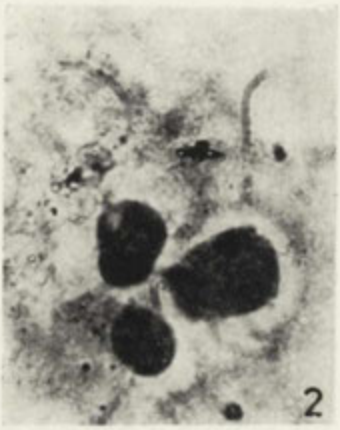
EXPLANATION OF PLATE I

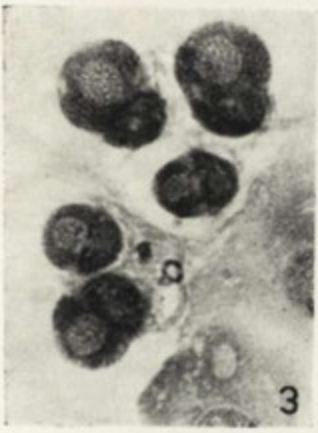
Steinina lunata sp. n.

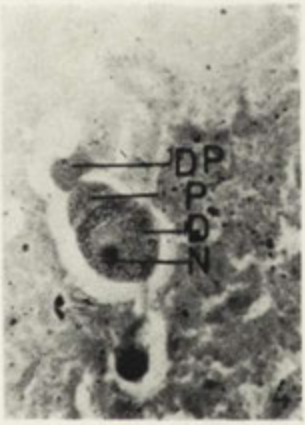
1: Earliest stage of the protozoan × 1000
2, 3: The body is partitioned by a septum into deutomerite anterior protomerite and posterior deutomerite × 1000
4: Cephalont × 1000
5, 6: Sporont × 1000
7: Cyst × 400

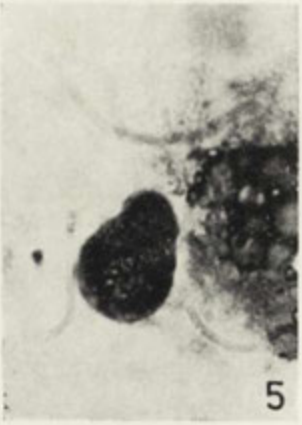
Deutomerite (D), Digital process (DP), epimerite (E), protomerite (P), nucleus (N)

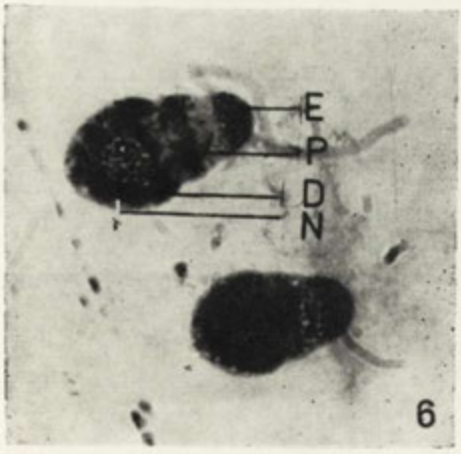


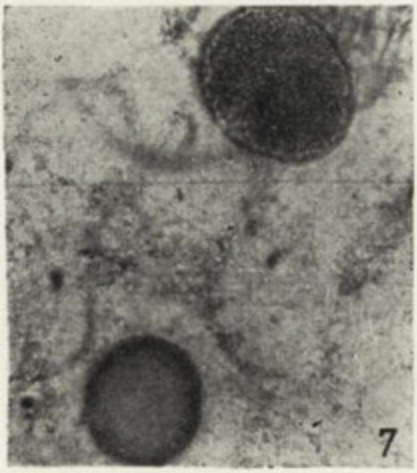












D. Kaur et P. Bala auctores phot.

NOTICE TO AUTHORS

ACTA PROTOZOOLOGICA is intended as a journal serving for the publication of original papers embodying the results of experimental or theoretical research in all fields of protozoology with the exception of faunistic notices of the local character and purely clinical reports. The papers should be concise as will not be accepted if they have been previously published elsewhere. After acceptance by the Editors papers will be printed in the possibly shortest time.

Papers are accepted in English, French, German and Russian. Every paper should begin with the name and postal address of the laboratory, name and the surname of the author and title in the language of the text. The titles in German and French should be translated into English according to the demands of Current Contents. The paper should be accompanied by synopsis in the language of the text not exceding 100 words and by short summary in one of 4 languages accepted in the Journal. In the Russian text also the name and the postal address of the laboratory, legends of tables, plates and text illustrations must be translated, the translation of the summary may be somewhat more extensive, and the name of the authors should be given additionally also in the Latin characters.

Manuscripts should be doublespaced typescript (30 lines one side of a sheet) with a normal margin. No elements of the text should be fully typed in capitals nor in spaced set (only underlining with pencil is admittable). In decimal fractions points not commas should be used. The generally accepted abbreviations and symbols are recommended. Nomenclature must agree with the International Code of Zoological Nomenclature, London 1961. The original and one carbon copy of the whole text material should be supplied.

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