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Genetic Analysis of Ciliary Pattern Mutants in *Paraurostyla weissei*

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Synopsis. Genetic analysis of inbred strains of the hypotrich ciliate *Paraurostyla weissei* revealed, that the phenotype of previously described (Jerka-Dziadosz 1989) variant line 95 is under control of two recessive genes: *mlm* (multi-left-marginal) and *pl* (pattern lability). In the course of this study three classes of abnormal phenotypes were isolated, all showing coordinated changes in the cortical pattern of ciliary structures.

The expression of ciliary pattern abnormalities shows transition from rare duplication of the left marginal ciliature in *pl* mutants to a significant increase in the multiplications of the ciliature at the left side in *mlm* mutants. In the doubly recessive *mlm/pl* mutants, in addition to the *mlm* syndrome the multiplication of the frontal, ventral, transverse and right marginal and dorsal ciliature as well as defects in the oral ciliature are expressed.

Comparison of cortical patterns in different doubly recessive *mlm/pl* lines revealed that they all express variant states of the original *mlm* phenotype (Jerka-Dziadosz and Banaczyk 1983) intensified by the *pl* gene. Different genotypes form a series of progressively stronger phenotypes, where the expression of the deviations from normal ciliary pattern is gradually amplified. The degree of expression of the *mlm/pl* mutations is dependent on environmental conditions and genic background.

In ciliated protists mutations expressed in alteration of the pattern of ciliary structures can be divided into three major groups: one class expresses small deletions in pattern elements (basal bodies, membranelles) without serious modifications in the overall pattern (Frankel 1973, Ruiz et al. 1987), the second expresses abnormalities in specific cor-

tical domains — the oral ciliature (Frankel et al. 1984 b, c, Lansing et al. 1985, Williams and Hont 1987), the third comprises mutants in which there are long-range coordinated changes in the overall pattern (Cole et al. 1987, Dubielecka and Jerka-Dziadosz 1989, Frankel and Nelsen 1986, Frankel et al. 1984 a, Jerka-Dziadosz 1989, Jerka-Dziadosz and Frankel 1979).

Paraurostyla weissei is a large hypotrich ciliate with the ciliature diversified into site specific sets of compound ciliary structures such as the frontal, ventral marginal, transverse cirri and oral membranelles located at the ventral surface (Pl. I 1), and two sets of dorsal bristles marking the dorsal side. The diversity of the body ciliature makes this organism a unique object for the study on pattern formation, since each body sector has its marker in the form of specific, cortically located ciliary structure.

In *P. weissei* the sexual process involves the total conjugation (Heckmann 1965), in which the two mates fuse and all ciliary structures are resorbed (Jerka-Dziadosz and Janus 1975). After fertilization a zygocyst is formed. In the zygocyst the development of the new macronuclear anlagen takes place, 8-10 days after cell union the primordia of ciliature are formed and a single exconjugant develop. This species, therefore, offers the opportunity to study the acquisition of cortical pattern abnormalities after conjugation when the ciliature is formed anew, without constraints imposed by the preexisting ciliature.

By extensive inbreeding, we isolated several cell lines in which the positioning of primordia was modified (Jerka-Dziadosz 1989, Jerka-Dziadosz and Banaczyk 1985). Detailed analysis of development of ciliary primordia in the single-gene recessive mutant *mlm* of *P. weissei* (Dubielecka and Jerka-Dziadosz 1989) revealed modifications in morphogenesis comprising the excessive production of basal bodies in the left half of the ciliate. In extreme cases, the mutant develops double sets of the oral primordia, multiple streaks of the left marginal cirri, and more than one primordial row in the left dorsal rows of bristles. Thus in *mlm* mutants widening and overlapping of longitudinal cell sectors competent for the initiation of normally separate sets of ciliary primordia occurs.

The *mlm* syndrome occurs also in another segregant, line 95 (Jerka-Dziadosz 1989), where in addition to the *mlm* syndrome, variability and/or complete lack of the right marginal cirri and overproduction of the right dorsal bristles was found.

Since in line 95 the *mlm* syndrome was enhanced and slightly modified we initiated an investigation aimed at establishing the genic background of this mutant. We suggest that the phenotype of line 95 is

caused by joint action of two recessive genes: *mlm* and *pl* (pattern lability).

Material and Methods

Ciliates employed in this study were clonal cultures of stocks collected from several natural ponds in Poland as well as progeny of crosses between different lines. Phenotypes comprised both normal single cells and experimentally obtained mirror-image doublets (Jerka-Dziadosz 1983) and multi-left-marginal mutants (*mlm*) (Jerka-Dziadosz and Dubielecka 1985).

A mirror-image doublets was obtained after subjecting a growing culture of singlets from line 20 to heat treatment. Well fed cultures grown at room temperature (approximately 22°C) were transferred to an incubator at 34.5° for 48 h and fed regularly. The cells grew very large and bulky and cell division was arrested. Cultures were transferred back to room temperature and 5 h later a semi-synchronous divisional morphogenesis took place. The culture was then transferred back to the incubator for 10 min in 40°C. Cell division was arrested in many cells, and monsters composed of non-separated division products appeared. Such monsters were isolated singly into depression slides. During subsequent days, isolates were checked for the presence of mirror-image doublets. One such doublet was isolated and grown clonally (Jerka-Dziadosz 1985). The genotype of the mirror-image doublet was the same as that of the original singlet line from which it had been produced (Fig. 1), that is, it was heterozygous at the *mlm* locus.

The origin of the multi-left-marginal mutant (*mlm*) was described previously (Jerka-Dziadosz and Banaczyk 1983), *mlm* lines appeared in the progeny of inbreeding crosses. The *mlm* phenotype is determined by a single recessive gene *mlm* (Jerka-Dziadosz and Dubielecka 1985). The methods of cultivation, induction of conjugation and isolation of exconjugants were the same as described previously. Samples were stained with silver proteinate (Protargol, Merck) according to protocols described elsewhere (Jerka-Dziadosz 1985).

Statistic tests were performed by the Computing Laboratory of the Nencki Institute.

Results

Genetic Study

The line 95 mutant was obtained in an experiment originally designed to isolate a mirror-image doublet line homozygous at the *mlm* locus. Mirror image line 20 (heterozygous at the *mlm* locus) was crossed to *mlm* line B-33. After mixing of the two cultures both homotypic (doublet × doublet) and heterotypic (doublet × singlet) pairs were isolated into depression slides and the progression of the conjugation process was observed daily. From 102 isolated pairs 24 produced zygocysts and developed into exconjugants. Seven exconjugant doublet cells and six single exconjugants died shortly after development of ciliary structures. Eleven

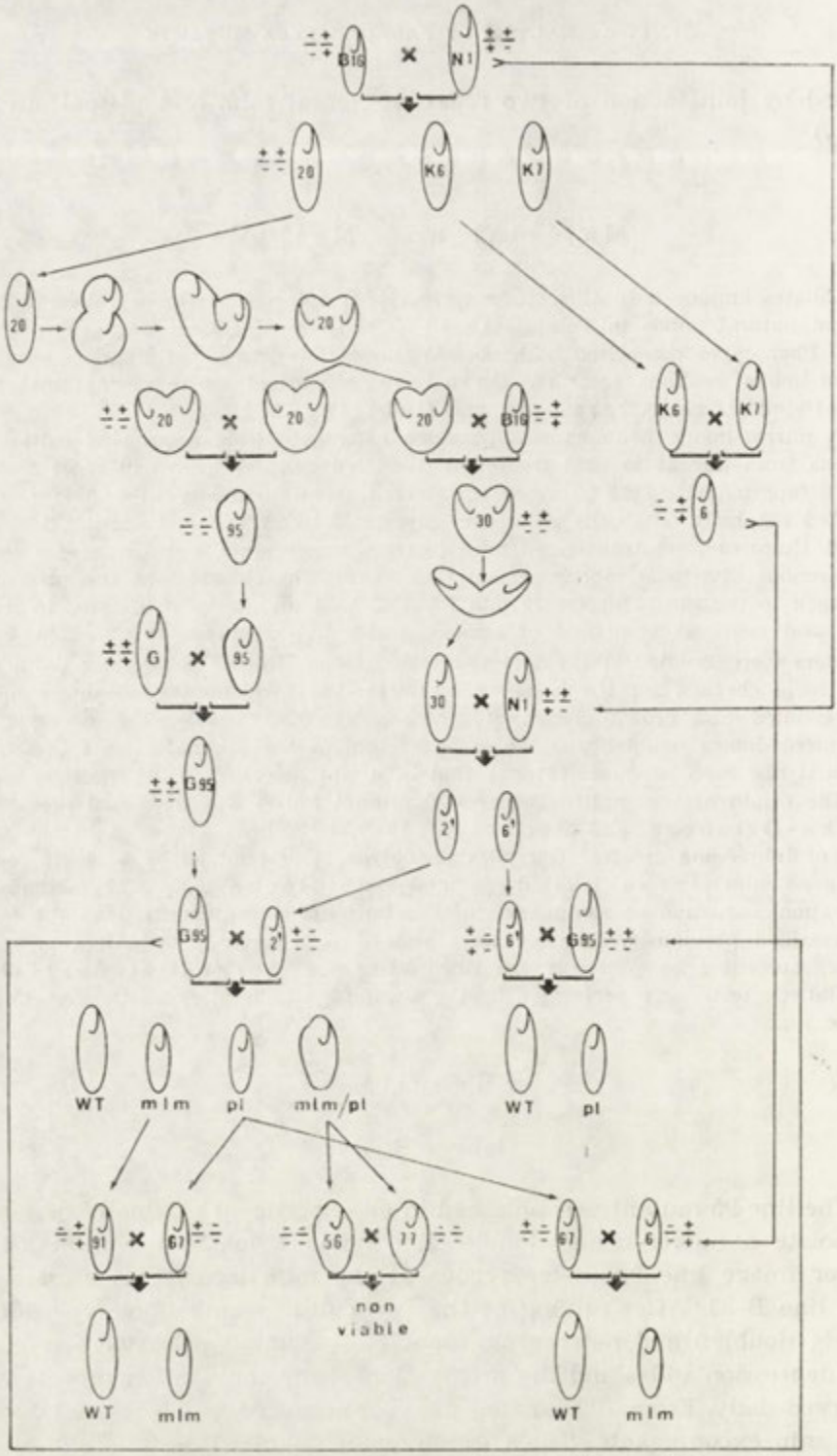

Fig. 1. A schematic representation of a cross between different lines of *P. weissei*. The designations of stock are indicated within the cell outlines. The presumed genotypes are indicated next to the cell outlines. In each case the first symbol represents the *mlm*, the second *pl*; genotypes of the first cross genotypes are: $mlm - pl +$ $+ /mlm - pl + \times mlm + pl + /mlm + pl -$

single exconjugants grew further forming clones of single cells. From these 6 showed wild-type morphology, 5 showed slightly abnormal shape. One of the exconjugant lines (No. 95) which originated from total conjugation of two doublet cells (Fig. 1) showed a peculiar cell shape: the right dorsolateral side of the cells were distinctly humped. This line appeared to be a mutant line showing the *mlm* syndrome and additional abnormalities in the right marginal cirri and right dorsal bristle rows. The abnormalities in the cortical pattern were described elsewhere (Jerka-Dziadosz 1989).

Line 95, after attaining sexual maturity (about 1 year after conjugation) was crossed to 7 lines having different phenotypes, including mirror-image doublets and *mlm* lines. Pairs were readily formed but the survival after conjugation was very poor (less than 5%) due to extensive inbreeding. Only one progeny line from cross 95 × G (G was a wild type line) was obtained and attained sexual maturity about 1 year after establishment. This line (G95) showed a wild type cortical pattern.

From analysis of the pedigree of previous crosses it appeared that the second gene locus (*pl* — pattern lability) involved in the phenotype of line 95 was probably present in the phenotypically wild type line N1 used in the genetic analysis of the *mlm* locus (Jerka-Dziadosz and Dubielecka 1985). Line N1 was therefore crossed to lines 95 and to the doublet line 20 but no progeny survived. In a parallel cross, line N1 was crossed to a presumably doubly heterozygous doublet line 30 (a progeny of doublet 20 × B16) (Fig. 1). Among the progeny of that cross, some lines showed slower growth rate (one division per day or less) and slightly abnormal ciliary pattern. Two lines: 2' and 6' were studied further. Both showed a slight decrease in growth rate. Some cells from line 2' possessed short additional rows at the left margin and the right marginal row appeared interrupted. In line 6' no significant alterations in cortical pattern were observed. It was presumed that both lines: 2' and 6' were homozygous at the *pl locus*, while line 2' was shown by further breeding to be heterozygous at the *mlm* locus.

Both lines 2' and 6', after attaining sexual maturity (about 4 months) were crossed to line G95 (Fig. 1). The result of that cross is shown in Table 1. The initial screening for abnormal cells was based on the observations of growth rate and changes in the cells shape. When cultures of all surviving progeny lines were established, samples were stained with protargol. In the first cross (G95 × 6') 12 fast growing progeny lines (2 divisions per day) had no deviations in cortical pattern and were thus considered as normal wild-type. The cells in the remaining 12 lines grew more slowly, were smaller and showed some deviations in morphology. In a small percentage of the cells, an additional short row of cirri

Table 1

Genetic interpretation of the genotype of line 95

Cross	Genotypes of parents	Experimental results phenotypes		Theoretical value	Difference
G95 × (30 × Nl)6′	$\frac{mlm+\ pl+}{mlm-\ pl-} \times \frac{mlm+\ pl-}{mlm+\ pl-}$	WT	12	12	0
		pl	12	12	0
G95 × (30 × Nl)2′	$\frac{mlm+\ pl+}{mlm-\ pl-} \times \frac{mlm+\ pl-}{mlm-\ pl-}$	WT	30	23.6	6.4
		pl	16	23.6	7.6
		mlm	10	7.9	2.1
		mlm/pl	7	7.9	0.9
(G95 × 2′)91 × (G95 × × 2′)67	$\frac{mlm-\ pl+}{mlm-\ pl+} \times \frac{mlm+\ pl-}{mlm-\ pl-}$	WT	14	12	2
		mlm	10	12	2
(K6 × K7)6 × (G95 × × 2′)67	$\frac{mlm-\ pl+}{mlm-\ pl+} \times \frac{mlm+\ pl-}{mlm-\ pl-}$	WT	24	23.5	0.5
		mlm	23	23.5	0.5

was present at the left margin and one elongated macronucleus instead of two oval ones occurred occasionally. In this cross, no cell line was found with the full *mlm* syndrome.

In the second cross (G95 × 2'), the survival after conjugation was very good. One hundred pairs were isolated and observed daily. Seventy three percent of pairs formed zygocysts and developed into exconjugants, 10 exconjugants died after one or two divisions. Thirty lines grew very quickly (2 or more divisions per day) and were therefore considered normal and discarded. The remaining 33 lines were all stained and the phenotypes were classified. Three phenotypes were found (Table 1).

(1) Lines slightly abnormal, with similar abnormalities as those found in the half of the progeny of the previous cross.

(2) Lines with clear *mlm* syndrome.

(3) Lines with grossly enhanced *mlm* syndrome as in line 95. The segregation of the phenotype fits the 3 : 3 : 1 : 1 ratio ($P > 0.05$) supporting the hypothesis that their phenotype is under control of two recessive genes: *mlm* and *pl*.

Since all three groups of phenotypes show the multi-left-marginal syndrome to a different degree, it was possible that the *mlm* and *pl* genes are in fact allelic. To test this possibility we crossed *mlm* and *pl* stocks to each other (Fig. 1). In the first cross line 91 and 67, progenies from cross G95 × 2' were mixed. From 100 isolated pairs 43% died after zygocysts formation. From 57 exconjugants only 24 grew and pro-

duced cultures. The rest did not divide or died after one division. In about half of the culture clones obtained in this cross we observed a decline in the fission rate after several days of cultivation. These lines showed the *mlm* phenotype. The other half (Table 1) showed the wild-type phenotype.

Line 67 was also crossed to *mlm* 6 a mutant line obtained in previous crosses (Dubielecka 1987). In this cross we isolated 61 pairs, from which 14 did not survive the zygocyst stage, the rest of the progeny, again, showed a segregation ratio 1 : 1 of wild-type and *mlm* lines. The results of these two crosses, first, excluded that *mlm* and *pl* mutants are allelic, and secondly, confirmed the genotypes assigned to the mutant lines previously used in crosses. Results of other crosses (data not shown) also confirmed this conclusion.

In order to test the variability of expression of the doubly homozygous *mlm/pl* phenotype several attempts to cross different *mlm/pl* lines with each other were made. In a cross 56 × 77, 100 pairs were isolated, only 16 exconjugants were rescued, from these 10 died without dividing, 5 divided once and then perished, one exconjugant divided three times and then died. In another cross (56 × 20) 50 zygocysts were isolated and all died. These results of crosses of doubly homozygous lines are similar to the results of crossing homozygous *mlm* lines to each other (Jerka-Dziadosz and Dubielecka 1985, Dubielecka 1987 (PhD Thesis)), where the progeny of such crosses also did not survive.

Analysis of Phenotypes

From stained samples of progeny lines of cross G95 × 6' and G95 × 2' we randomly selected one line from each phenotype for further detailed analysis. The following lines were chosen: from the first cross: line 18 a (wild type) and line 3 c (*pl* — slightly abnormal), from the second cross line 53 (*pl* — slightly abnormal), line 12 (*mlm* — multi-left-marginal) and line 56 (*mlm/pl* — pervasive ciliary multiplication). In non dividing cells, the length of cells was measured and the number of frontal transverse and right marginal cirri was counted in 20 cells from each sample. We also scored the number of ventral cirral rows and the number of the left and right marginal cirral rows. The data are presented in Table 2 and Fig. 2).

The wild type line 18 a differs from the remaining abnormal lines in the stability of the number of frontal cirri and the stable single row of left and right marginal cirri (Pl. I 1).

The phenotype of *mlm* line 12 analyzed in this study fits within the range of abnormalities found in other *mlm* lines, described in detail pre-

Table 2

The number of structures in different lines of *P. weissei*

No. of line	FC	TC	VC	LM	RM	Phenotype
	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%	
18 a	4–4 4.0 0 0	7–10 8.44 0.71 8.41	4–6 5.08 0.40 7.87	1–1 1 0 0	1–1 1 0 0	WT
12	4–6 4.1 0.45 10.98	6–9 7.25 0.64 8.83	4–5 4.5 0.51 11.33	1–3 2.35 0.67 28.51	1–1 1 0 0	*mlm*
3 c	3–4 3.92 0.27 6.88	6–9 7.56 0.71 9.39	4–5 4.88 0.33 6.76	1–2 1.24 0.43 34.6	1–1 1 0 0	*pl*
53	3–4 3.84 0.37 9.63	6–8 6.88 0.44 6.39	4–5 4.16 0.37 8.89	1–2 1.04 0.20 19.23	1–1 1 0 0	*pl*
56	3–6 4.39 0.72 16.40	7–14 9.95 1.79 17.98	5–10 7.17 1.07 14.92	1–3 2.00 0.60 30.00	1–3 1.91 0.79 41.30	*mlm*/*pl*

viously (Dubielecka and Jerka-Dziadosz 1989, Jerka-Dziadosz and Banaczyk 1983).

The two *pl* lines (3 c and 53) do not differ from each other with respect to the frequency distributions of the studied parameters, except the more frequent occurrence of cells with 5 VC rows in line 3 c than in line 53. Occasionally, cells with two left marginal cirral rows are observed. Usually the second, short row is located in the posterior half of the cell, whereas the first row appears similar to the LM row in wild-type cells. The ciliature of the right margin of *pl* lines is comparable to that in wild-type and *mlm* lines. They all possess one row of cirri at the right margin. In developing cells, abnormalities other than in the LM primordia, and very rarely in dorsal kinety I primordia, were not observed.

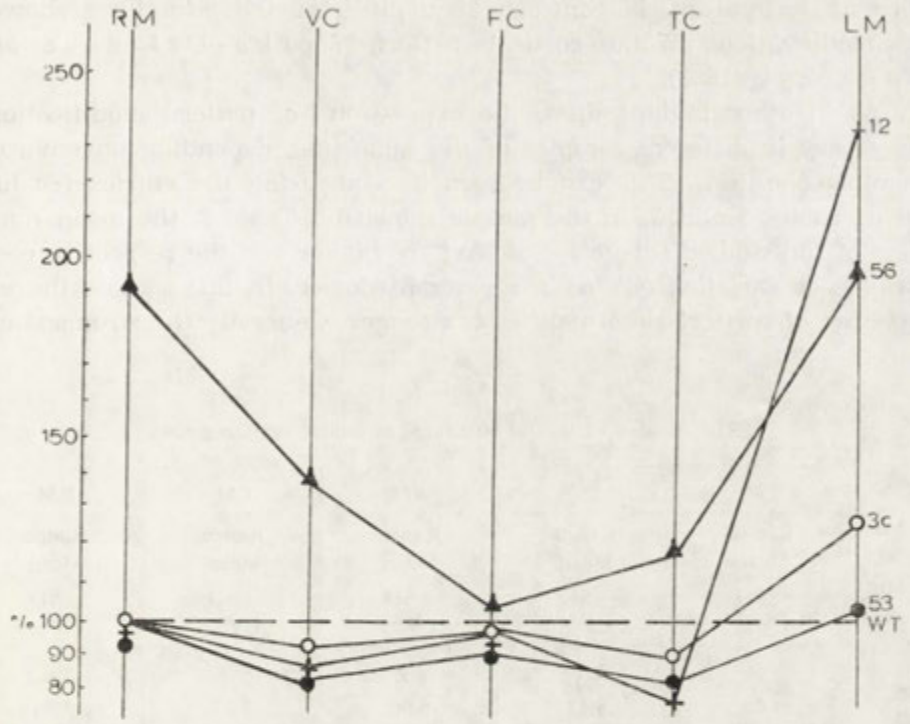

Fig. 2. A graphic comparison of the relative mean number of the studied categories of cirri in cell lines expressing different phenotypes (compare with table 1). The mean numbers of the studied structures in wild-type cell line 18 a are represented as 100% (the broken line of the abscissa). The corresponding mean values in the mutant lines are indicated as percentages of the wild type mean value of the number of a particular cirral structure

When compared to wild-type cells, *pl* cells (Pl. I 2) appear a bit smaller and possess somewhat fewer frontal and transverse cirri. The mean size of populations (3 c — 131.88 μm, SD ± 10.99; 53 *pl* — 164.36 μm, SD ± 10.10) fits within the range of *mlm* lines (e.g., 142.70 μm in line M7, (Dubielecka and Jerka-Dziadosz 1989). About 30% of *pl* cells possess 1 elongated macronucleus (Pl. I 2) instead of two ovoid nodes. Cultures of established *pl* lines differ from wild-type clones only by their slightly smaller size, and slightly slower growth rate. From *mlm* cultures they differ in the lack of rounded up monsters and less frequent reorganizing cells.

In the *mlm/pl* line 56 there are generally more ciliary structures present on the ventral surface than in the wild-type, *pl* and *mlm* lines (Table 2). In addition, the variability in number of all structures is also remarkably greater. In any sample of cells, a wide range of phenotypes can be seen while cells without modification in the cortical pat-

tern are very rare, in contrast, about 10% of the *mlm* lines showed no modifications in the cortical pattern (Jerka-Dziadosz and Banaczyk 1983).

As in other mutant lines, the expression of pattern modifications may vary in different samples of the same line, depending on environmental conditions. This can be seen by comparing the entries for line 56 in Tables 2 and 3. In the sample shown on Table 3, the mean numbers of the studied categories of cirri are higher and the respective coefficients of variation (CV%) are generally lower. In this sample the expression of cortical abnormalities is stronger. Generally the strongest ex-

Table 3

The number of ventral structures in double homozygotes

No. of line	FC	TC	VC	LM	RM
	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%	Range Mean ±SD CV%
18	3–6 4.08 0.67 16.40	5–12 8.53 1.55 18.20	4–8 6.06 1.28 21.10	2–5 3.27 1.16 35.40	0.5–2 0.96 0.34 35.40
20	4–11 6.00 2.80 46.70	10–16 13.14 1.49 11.30	6–14 9.00 1.61 17.80	1–4 2.47 0.67 27.10	1–3 2.09 0.62 29.60
32	3–6 4.14 0.65 15.70	9–15 11.30 1.52 13.40	5–8 6.70 1.03 15.30	2–5 3.15 0.81 25.70	1–3 1.75 0.55 31.40
56	4–6 5.00 0.69 13.80	9–16 12.09 1.67 13.80	7–10 8.40 1.05 12.50	1–5 1.86 0.83 44.60	1–3 2.09 0.75 35.80
77	4–7 5.80 0.89 15.30	6–16 11.65 2.13 18.20	6–11 8.20 1.15 14.00	1–4 2.45 0.75 30.60	1–3 2.20 0.69 31.30
95	3–5 4.17 0.57 13.6	4–10 7.82 1.33 17.0	3–7 4.26 0.93 21.8	1–5 2.34 0.93 39.7	0–2 0.82 0.46 56.0

pression is seen in well-fed and fast growing cultures, in which the cells are relatively large.

At the left margin in cells from line 56, there are 1-5 rows of marginal cirri. Usually there are 1-2 long rows covering the whole left margin extending from the AZM toward the posterior end, curving around the posterior end and terminating at the posterior right side of the ciliate. The shorter rows can be located on either side of the longer ones, and frequently overlap each other. The LM rows are more densely spaced than in *mlm* lines. This is probably related to crowding (Pl. I 3) of numerous ventral structures on a similar ventral area.

The double homozygotes possess abnormalities also in the adoral zone of membranelles (Pl. II 6). In about 40% of morphostatic cells of line 56 a defect in the AZM occurs. Usually in the middle of the proximal part of the AZM there is a gap in the membranelles. Posterior to the gap a group of disorganized membranelles located to the left of the proper AZM is seen. This group, variable in size, appears to be a remnant of an expanded oral primordium seen in many developing cells (Jerka-Dziadosz, unpublished observations). Unlike *mlm* lines where the additional oral primordia are resorbed (compare Fig. 4 in Dubielecka and Jerka-Dziadosz 1989), in *mlm/pl* lines this expanded OP differentiates into membranelles which cannot be incorporated into a normally functioning AZM and they remain as pieces of a secondary AZM.

The paroral membranes (UM) in the light microscopic preparations appear normal. Duplication of UMs was not observed.

The main part of the ventral ciliature: the frontal, ventral and transverse cirri are greatly modified in the doubly homozygotic lines. Modifications comprise both their number and morphology. In line *mlm/pl* 56 the number of frontal cirri located immediately posterior of the frontal part of the AZM ranges from 3-6 (Table 2). Usually there are 4 large cirri corresponding in location to those in a normal cell, and 2 additional cirri. In other *mlm/pl* lines the number of additional cirri reaches 11 (compare Table 3, line 20). Usually two additional cirri are located in the frontal area anteriorly to the UM (Pl. II 4, 5) or to the left of it. Often only remnants of the additional cirri are visible, as they frequently become resorbed, leaving some of the accessory microtubular rootlets and a modified subpellicular cytoskeleton (Pl. I 3). Sometimes the additional frontal cirrus No. 1 is rotated by 90° or 180° in the horizontal plane (Pl. II 5). In some cells pictures suggesting a fusion of two frontal cirri located close to each other were seen. Both resorption and fusion of frontal cirri causes the incidence of additional FC to be less frequent on predivision cells than in young cells. In developing cells, the newly formed additional FC are very prominent in all *mlm/pl* lines isolated in this study.

The number of ventral cirral rows varies from 5-10 (4-6 in normal cells). The ventral rows are more densely spaced than in normal cells (Pl. I 3). The general pattern of ventral rows is comparable to normal cells. The rows located on the left side end near the middle of the cell, the remaining rows terminate near the transverse cirri. The length of ventral rows is variable. Some start at the right side of the frontal area, others in the middle of the cell. In many adult cells, the ventral rows are oriented slightly obliquely. The individual ventral cirri appear to be smaller than in wild-type cells.

The transverse cirri, similarly to FC, differ in number and morphology. The number is significantly higher, but the general location is similar to that in normal cells. The morphology of TC is not uniform. There are large TC with typical appearance, as well as small ones pushed in between them (Pl. II 7). Some appear to be in the process of resorption, others fuse one with another.

The ciliature of the right margin of the line 56 shows a range of variability similar to that of the ciliature of the left margin. The number of RM rows ranges from 1-3, but the total number of cirri (34.5) distributed into the rows is smaller than in normal cells (42.84). The cirri may be arranged as one long row (containing 19-41 cirri), as two rows, one long and one short (e.g., 30 + 3), as two short ones (e.g., 7 + 7) or as three short rows (e.g., 14 + 12 + 14). Usually, shorter rows are located in the posterior part of the cell. Frequently, the multiple RM rows are arranged in an overlapping mode. In contrast to normal cells, there is no clear gap between the right ventral and right marginal cirri, the transition is gradual (Pl. I 3). In contrast to the original *mlm/pl* line 95 described previously (Jerka-Dziadosz 1989) cells lacking the right marginal cirri were not found in line 56.

The dorsal ciliature was not studied in detail in morphostatic cells. In developing cells multiplication in the number of primordial rows were observed in both the left and right dorsal rows of cilia. Differentiation of dorsal bristles at the anterior termini of the left marginal cirral rows occurs in some cells (Pl. I 3). These modifications appear similar to those found in *mlm* lines (Dubielecka and Jerka-Dziadosz 1989).

Variability in the Expression of the Pervasive Multiplication of Ciliary Structures

From the cross G95 × 2' we rescued seven lines expressing the pervasive ciliary multiplication syndrome (Table 2). The phenotypes of the double homozygotes were analyzed in 5 lines: 18, 20, 32, 56 and 77

(Table 3). A statistical test (Kruskal-Wallis) analyzing the average ranks of all studied parameters revealed that for each parameter the cell lines differ significantly. None of the lines studied resembled the original line 95 in all respects. (Table 3, Fig. 3).

Line 18 was the most similar to 95 in such parameters as the number of frontal, transverse and right marginal cirri. However, the number of LM and ventral cirral rows was remarkably higher. About 25% of cells from line 18 possessed a single right marginal row shorter than normal, covering only the posterior half of the margin.

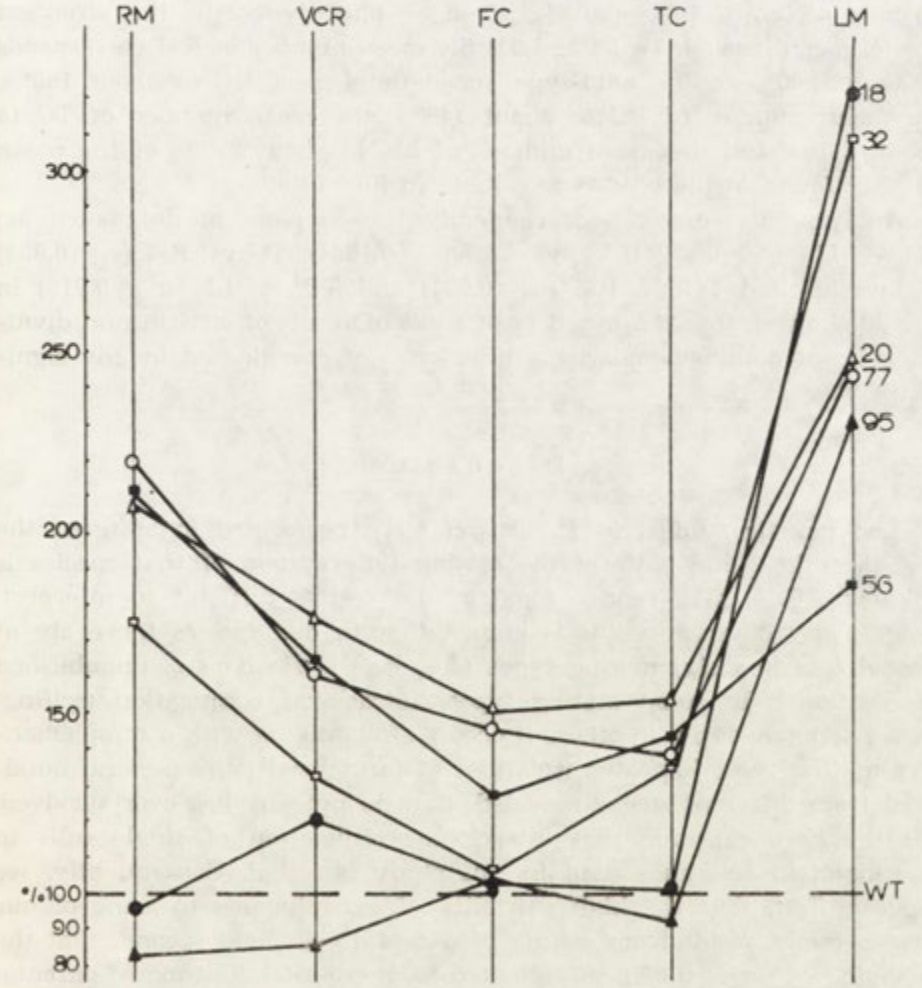

Fig. 3. A graphic comparison of the relative mean numbers of the studied categories of cirri in double homozygotes *mlm/pl/mlm/pl*. In relation to the wild-type line 18 a (broken line of the abscissa, compare with Table 3). Conventions as on Fig. 2

Line 32 expressed relatively small modifications in the frontal and transverse cirri. The multiplication of left marginal cirral rows was expressed very profoundly, and the number of right marginal cirri ranges from 1 to 3 (Pl. II 3).

The remaining three *mlm/pl* lines 56, 77 and 20 show a similar general character of expression of the modified phenotype. In line 20 modifications in VC, FC and TC are expressed most strongly (Fig. 3, Table 3).

The mean numbers of structures, expressed in percentages of means calculated for the wild-type line 18 a (Table 2), represented in Fig. 3 form a series of progressively stronger phenotypes. In the strongest phenotypes (lines 20, 77, 56 and 32) the mean number of RM corresponds to about 200% of the wild-type, mean number of VC to about 160%, the mean number of FC to about 140%, the mean number of TC to about 150%, and the mean number of LM to about 270% of the mean number found in the wild-type cells from line 18 a.

Analysis of regression of randomly chosen pairs of data such as: RM vs. LM ($r = 0.1821$), VC vs. LM ($r = 0.1684$), TC vs. RM ($r = 0.031$) in line 32, and TC vs. RM ($r = 0.091$) and TC vs. LM ($r = 0.218$) in line 20 showed, that the numbers of particular sets of cirri in non dividing cells of doubly homozygous lines are not coordinated in any significant way.

Discussion

The genetic studies in *P. weissei* were performed in spite of the fact that the genic basis of the mating type system of that species is unknown. This may render some of the results difficult to interpret. From many observations it is known that in our species there are at least 4 (possibly six) mating types (Jerka-Dziadosz, unpublished observations). In some mating types, intraclonal conjugation (selfing) occurs spontaneously, in others it occurs after mixing with a complementary mating type. Repeated isolations of intraclonal pairs (several hundreds) from different stocks revealed, that no progeny has ever survived. We therefore presume that intraclonal conjugation of single cells in *P. weissei* in cell lines used in this study is lethal. Consequently, we suggest, first, that the high mortality of exconjugants in some of our crosses might result from selfing of parental cells, and second, that the surviving exconjugant lines originated from cross-fertilization of parental cells.

We found two exceptions from this general rule. One is a clone showing permanent selfing in clonal cultures, isolated from a pond in Mün-

ster (FRG) where about 20% of exconjugants produced viable, selfing, progeny lines. (This line was not used in this study). The other exception is the intraclonal conjugation of mirror-image doublet cells, occurring after mixing a doublet culture with a complementary mating type single cells, such as took place in the cross between double 20 and *mlm* B-33. In this cross two singlet exconjugants of selfing doublets were isolated. One of them was the mutant line 95 (Fig. 1).

The nuclear events taking place in the doublet during conjugation are not fully understood, and fusion of old and new macronucleus is not completely ruled out. The genetic study confirmed that at least the micronucleus of the exconjugant contained only recessive alleles of genes *mlm* and *pl* (see below). Since the cortical structures are lost during the passage through the zygocyst stage (Jerka-Dziadosz and Janus 1975), it seems safe to conclude that the mirror-image double state of the parentage of mutant *95* has no effect on the cortical pattern of the segregant.

It is worth to mention that, generally, the mirror-image double state is passed through the zygocyst stage to small percentage of the exconjugants. In a separate study (Jerka-Dziadosz, unpublished), from 178 pairs where one of the mates was a mirror-image doublet, only 3 exconjugant clones of doublets were obtained, 19 exconjugant doublets (10.6%) did not divide and died, the rest of the surviving progeny (45 lines) were single cells. The mirror-image doublet line 30 (Fig. 1) was one of the surviving doublets from these crossess. Thus in *P. weissei*, due to the decomposition of the ciliary structures during the zygocyst formation (Jerka-Dziadosz and Janus 1975) most exconjugants originating from doublets develop into single cells.

The main finding of this study is a demonstration that the phenotype of line 95 is under control of a joint action of recessive alleles at two loci *pl* and *mlm*. In the course of genetic analysis, three groups of abnormal phenotypes were isolated, all showing changes in the cortical pattern of ciliary structures (Fig. 2). The expression of cortical abnormalities in the three classes shows a transition from minor abnormalities occurring at the left side of the *pl* mutants, through a gradual increase in the abnormalities on the left side in multi-left-marginal mutants, up to a multiplication of the frontal, ventral, transverse and the right marginal cirri found in the double recessive (*mlm/pl*) mutants. The three classes of mutants form a series of progressively stronger phenotypes where the expression of defects is gradually amplified. The *pl* gene alone causes a very minor modifications in the cortical pattern, but it greatly intensifies the *mlm* phenotype, spreading the multiplications of ciliary structures rightward, embracing the whole ventral and dorsal sur-

face (Jerka-Dziadosz 1989). The result of the cross between *mlm* and *pl* lines (Table 1) where half of the progeny showed the wild-type phenotype established that the *mlm* and *pl* are not allelic, although phenotypically the *pl* lines sometimes express a very weak multi-left-marginal syndrome.

Analysis of the phenotypes of the double *mlm/pl* homozygotes isolated in this study has shown that in these mutants practically all categories of ciliary structures undergo multiplication (Fig. 3). In this respect these doubly recessive lines differ from the original double homozygote 95, where the mean number of the ventral cirri (4.26) was the lowest among the *mlm* lines (Dubielecka and Jerka-Dziadosz 1989) and was significantly smaller than in wild-type cells. In addition, in about 17% of cells there were no right marginal cirri present, in the remainder there were one or two short rows each consisting of smaller number of cirri than the normal cells. This last feature is enhanced in the *mlm/pl* lines 20, 77 and 56 where the number of short RM rows ranged from 1 to 3. The general pattern of arrangement of these RM rows was similar to that in line 95.

As concerns the number of ventral cirral rows, the number of frontal cirri and the number of transverse cirri they all are higher in *mlm/pl* lines isolated in this study than in line 95 (Fig. 3). It is worth recalling, that several dividing cells of line 95, where a multiplication of FVT streaks was observed, resemble very much dividing cells from other doubly homozygous lines.

The comparison of the modifications in morphogenesis of *mlm* lines and line 95 suggests conclusion that line 95 can be considered as a variant state of the expression of the *mlm* phenotype (Jerka-Dziadosz 1989). This study has shown that all other doubly homozygous lines (Table 3, Fig. 3) show stronger states of expression of *mlm*. It is possible that both genes act as mutual "enhancers" of each other, although such a hypothesis require a further experimental verification. The degree of expression of the *mlm/pl* mutations is dependent on environmental conditions and genic background. Mutual enhancement of phenotypes was described in *bicaudal* mutants of *Drosophila* (Mohler and Wieschaus 1986) in which the maintenance of the normal order of positional values is affected.

In the previous paper (Jerka-Dziadosz 1989) an interpretation was suggested that the pattern abnormalities found in the *mlm/pl* cortical mutants result from cyclic changes in the distribution of the ventral positional values and intercalation of the intermediate values at places of reiterations of positional values. Temporal aspects of expression of the variable positional values result in both widening and overlapping of longitudinal cell sectors competent for the initiation and

development of normally separate sets of the ciliary primordia. This widening and overlapping causes a particular primordium to develop in two adjacent cell sectors giving rise to multiple sets of ciliary structures in an adult ciliate. The dynamic character of the model will be further examined in developing cells of mutant lines isolated in this study where the instability of positional values is expressed on the whole surface of the cell.

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REFERENCES

Cole E. S., Frankel J. and Jenkins L. M. 1987: *b,c,d*: A mutation affecting the width of organelle domains in the cortex of *Tetrahymena thermophilia*. Wilhelm Roux Arch. Dev. Biol. 196, 421-433.

Dubielecka B. 1987: Genetyczna kontrola przestrzennego uporządkowania wzoru powierzchniowego u orzęska *Paraurostyla weissei*. Wpływ mutacji *mlm* na rozwój zawiązków struktur rzęskowych. PhD Thesis. M. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland.

Dubielecka B. and Jerka-Dziadosz M. 1989: Defective spatial control in patterning of microtubular structures in *mlm* mutants of the ciliate *Paraurostyla*. I. Morphogenesis in the multi-left-marginal mutant. Europ. J. Protistol., (submitted).

Frankel J. 1973: A genically determined abnormality in the number and arrangement of basal bodies in a ciliate. Dev. Biol., 20, 336-365.

Frankel J. and Nelsen E. M. 1986: How the mirror-image pattern specified by a janus mutation in *Tetrahymena thermophila* comes to expression. Devel. Genetics, 6, 213-238.

Frankel J., Jenkins L. M. and Bąkowska J. 1984 a: Selective mirror-image reversal of ciliary pattern in *Tetrahymena thermophila* homozygous for a janus mutation. Wilhelm Roux Arch. Dev. Biol., 194, 107-120.

Frankel J., Jenkins L. M., Bąkowska J. and Nelsen E. M. 1984 b: Mutational analysis of patterning of oral structures in *Tetrahymena*. I. Effects of increased size on organization. J. Embryol. Exp. Morph., 82, 41-66.

Frankel J., Nelsen E. M., Bąkowska J. and Jenkins L. M. 1984 c: Mutational analysis of patterning of oral structures in *Tetrahymena*. II. A graded basis for the individuality of intracellular structural arrays. J. Embryol. Exp. Morph., 82, 67-95.

Heckmann K. 1965: Total Konjugation bei *Urostyla hologama* sp. n. Arch. Protistenk., 108, 11-38.

Jerka-Dziadosz M. 1983: The origin of mirror-image symmetry doublet cells in the hypotrich ciliate *Paraurostyla weissei*. Wilhelm Roux Arch. Dev. Biol., 192, 179-188.

Jerka-Dziadosz M. 1985: Mirror-image configuration of the cortical pattern causes modifications in propagation of micro-tubular structures in the hypotrich ciliate *Paraurostyla weissei*. Wilhelm Roux Arch. Dev. Biol., 194, 311-324.

Jerka-Dziadosz M. 1989: Defective spatial control in patterning of microtubular structures in mutants of the ciliate *Paraurostyla*. II. Spatial coordinates in double recessive mutant. Europ. J. Protistol., (submitted).
Jerka-Dziadosz M. and Banaczyk I. A. 1983: Cell shape growth rate and cortical pattern aberrations in an abnormal strain of the hypotrich ciliate *Paraurostyla weissei*. Acta Protozool., 22, 139-156.
Jerka-Dziadosz M. and Dubielecka B. 1985: Transmission of a genetic trait through total conjugation in a hypotrich ciliate *Paraurostyla weissei*. Genetic basis of the multi-left-marginal mutant. Genet. Res. Camb., 46, 263-271.
Jerka-Dziadosz M. and Frankel J. 1979: A mutant of *Tetrahymena thermophila* with a partial mirror-image duplication of cell surface pattern. I. Analysis of the phenotype. J. Embryol. Exp. Morphol., 49, 167-202.
Jerka-Dziadosz M. and Janus I. 1975: Discontinuity of cortical pattern during total conjugation of a hypotrich ciliate *Paraurostyla weissei*. Acta Protozool., 13, 309-333.
Lansing T. J., Frankel J. and Jenkins L. M. 1985: Oral ultrastructure and oral development of the misaligned undulating membrane mutant of *Tetrahymena thermophila*. J. Protozool., 32, 126-139.
Mohler J. and Wieschaus E. F. 1986: Dominant maternal effect mutations of *Drosophila melanogaster* causing the production of double-abdomen embryos. Genetics, 112, 803-822.
Ruiz F. N., Garreau de Loubresse N. and Beisson J. 1987: A mutation affecting basal bodies duplication and cell shape in *Paramecium*. J. Cell. Biol., 104, 417-430.
Wiliams N. E. and Hont J. E. 1987: The assembly and positioning of cytoskeletal elements in *Tetrahymena*. Development, 100, 23-30.

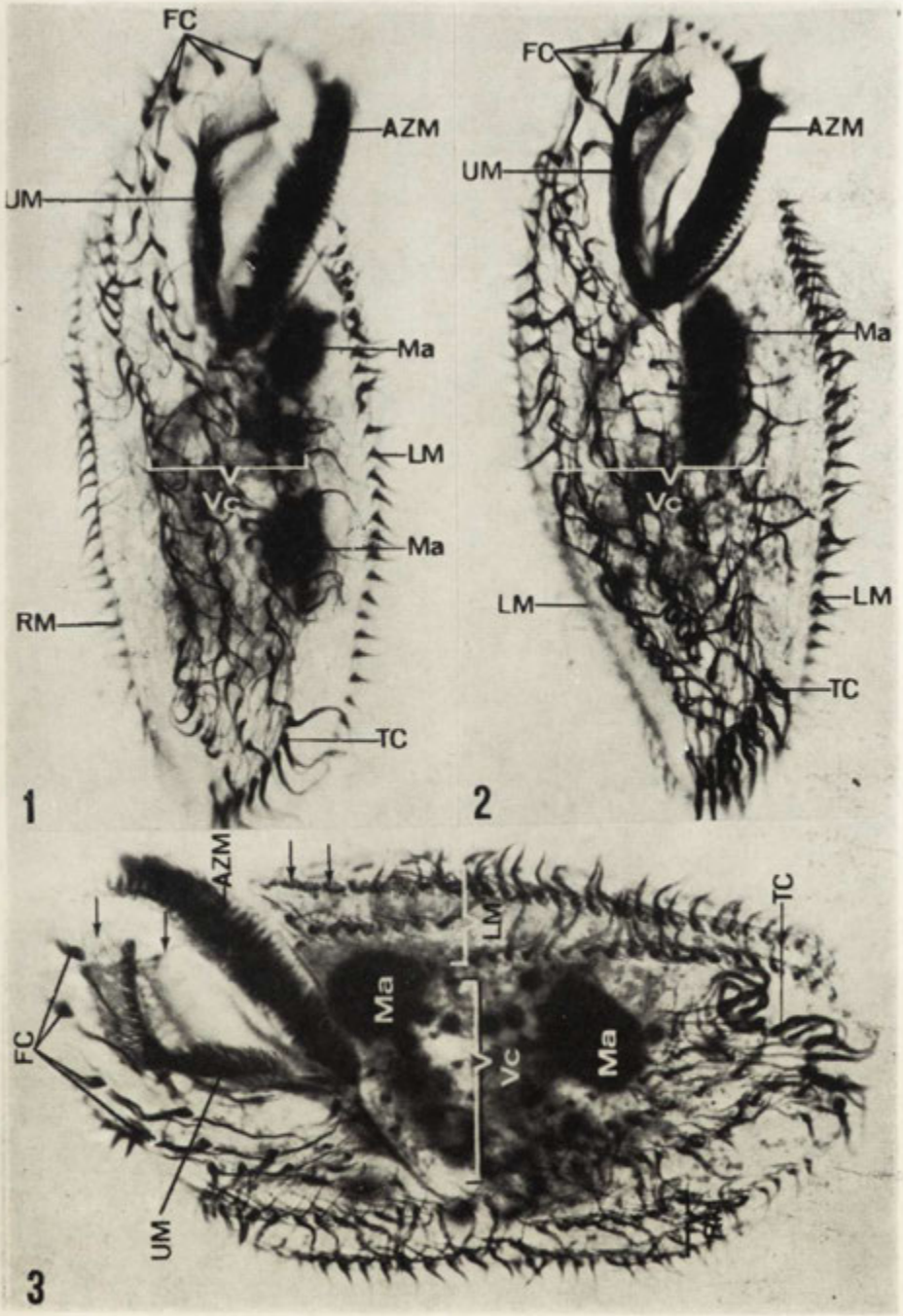
EXPLANATION OF PLATES I-II

The ventral surface of cells of *P. weissei* stained with protargol. The anterior of the cell is up.

1: A wild-type cell from line 18 a (900 ×)
2: A slightly abnormal cell from *pl* line 53. Note the shape of the cell and one elongated macronucleus (1100 ×)
3: A *mlm/pl* mutant cell from line 32. Note the multiple LM and RM rows. Arrows at the frontal area point to the remnants of the resorbed additional frontal cirri. The double arrow points to the anterior end of the LM where dorsal bristles develop
4: The frontal area from a cell of line 56. Note three cirri (arrows) at the place of frontal cirrus No. 1. The anterior microtubular derivatives are directed away from the AZM (asterisk) (1800 ×)
5: The frontal area of mutant cells from line 56. Normal cirri are indicated by numbers, the additional frontal cirri are indicated by arrows. The stars indicate the microtubular derivatives of the additional frontal cirri. Their orientation is changed as compared to the adjacent cirri. Arrows point to two additional cirri, note their smaller size (1400 ×)
6: The adoral zone of membranelles in a mutant *mlm/pl* from line 20. The bracket indicates an abnormal region of the membranellar band. The arrow points to the gap in the AZM (1850 ×)
7: The transverse cirri in a mutant cell from line 56. Arrows point to the fused TC (1450 ×)

Abbreviations

AZM — Adoral zone of membranelles, FC — frontal cirri, LM — the left marginal cirri, Ma — the macronucleus, *mlm* — multi-left-marginal, *pl* — pattern lability, RM — the right marginal cirri, TC — transverse cirri, UM — paroral membranelles, VC — the ventral cirral rows.



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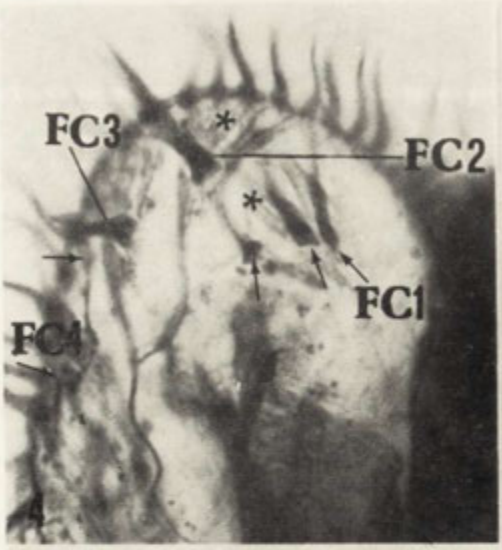

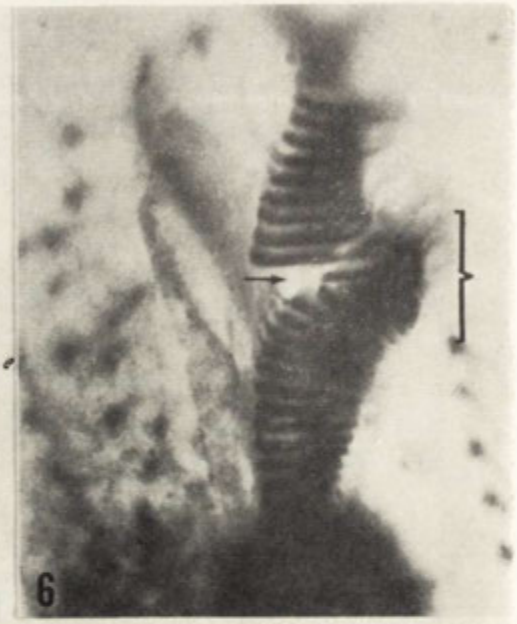

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Determination of Free and Bound Amino Acids in Three Strains of *Naegleria*

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Synopsis. The comparison of both bound and free amino acid patterns in three *Naegleria* strains showed great differences between them. In the free amino acid pools the major constituents were alanine and proline, while among the protein amino acids both glutamic and aspartic acids were the most abundant. Within the free amino acids only proline percentages were different for each strain ($P < 0.05$). Tyrosine values were higher in *N. gruberi* strains than in *N. lovaniensis*. The data obtained from the protein amino acids after five hydrolysis intervals are also compared.

After a careful review of the literature, we have found no publications about either free or constitutive amino acid determinations of any of the *Naegleria* strains presently known. In previous papers on three strains of *Naegleria* concerning different subjects (phagocytic activity, lectins and antigenic relationships) no greater similarities were found between two strains of *N. gruberi* than between each of these and *N. lovaniensis* (Alonso and Zubiaur 1985, Zubiaur and Alonso 1985, 1987). Therefore, in the present study a careful quantitative analysis of both free and protein amino acids in such amoebo-flagellates was performed to ascertain whether there were any quantifiable differences among these three strains.

Material and Methods

Strains and Culture Conditions

One strain of *Naegleria lovaniensis* (Aq/9/1/45D) and two strains of *N. gruberi* CCAP 1518/1e and 1518/1f), kindly supplied by Dr. J. De Jonckheere, were maintained axenically at 30°C in SCGYEM medium (De Jonckheere 1977).

Preparation of Extracts

Falcon flasks with each *Naegleria* strain from 96-h-old cultures (late log stage) were placed for 15 min in an ice bath to detach the cells from the walls of the vessels. The cells were centrifuged at 1500 g for 15 min and washed three times in PBS (0.01 M phosphate buffer, 0.15 M saline, pH 7.0), suspended in PBS and sonicated at 4°C in a MSE cell disruptor (Model Mk2, 150W), five strokes of 30 s, at a power setting of 20W. The disruption of the cells was checked using phase contrast microscopy. An aliquot was retained from the final wash for protein determination in order to detect any trace of protein that might remain from the culture medium.

In alternative assays, cells were disrupted after repeated freeze-thaw shocks.

Free Amino Acid Analysis

Prior to deproteinization an aliquot was taken from each homogenate for protein determination (Lowry et al. 1951). Homogenates were deproteinized as previously described (O'Daly et al. 1983) with slight modifications. In brief, protein elimination was carried out by precipitation mixing equal volumes of the homogenate and 10% (w/v) trichloroacetic acid (TCA) and centrifugation at 40 000 g for 30 min at 4°C. The supernatant fluids were passed through Millipore filters (0.22 μm pore size) to eliminate any remaining protein. The filtrates were extracted three times with diethyl ether to remove excess TCA. The final aqueous phases were lyophilized and further processed in a LC 7000 amino acid analyzer (Biotronic), complemented with a SP 4100 Spectra Physics computing integrator. A 3.2 mm column with Biotronic BTC 2710 resin was used. Readings were made at 570 nm; proline was checked at 440 nm as well.

The data derived from the integrator, after comparison with amino acid standard solutions, were expressed in nmol/50 μl. Such values, once transformed to nmol/mg protein were calculated as percentages for unification of results.

Protein Amino Acid Analysis

The homogenates obtained as previously described were dialyzed for 24 h against distilled water. Before hydrolysis a sample was taken from each dialysate for protein determination (Lowry et al. 1951). Aliquots of the dialysates, each containing 1 mg protein/ml were mixed in vacuum tubes with concentrated HCl to a final concentration of 6 N. The tubes once evacuated were kept at 104°C and subjected to hydrolysis for different time intervals (8, 16, 24, 48 and 72 h).

Hydrochloric acid was eliminated from the hydrolysates by dilution with distilled water which were kept in an oven at 60°C to dryness. This step was repeated several times until the acid was totally eliminated. As the vessels containing the hydrolysates were kept unstoppered, any cysteine present would be oxidized to cystine. The hydrolysates were then processed for analysis as described above for free amino acids.

Results

Free Amino Acids

The free amino acid pool amounted to about 800 nmol/mg protein for *N. lovaniensis* and about 600 nmol/mg protein for both *N. gruberi* strains. Table 1 shows the percentages of the individual amino acids for each strain. The mean values resulting from three separate experiments and the standard deviations (SD) are represented. Due to the high SDs obtained, Student's *t* test was applied looking for significance of the differences observed in the means. For the majority of the amino acids there were no significant differences among the three strains at the 5 per cent level. Only for proline the percentages were different for each strain ($P < 0.05$). In addition, tyrosine values were significantly higher in both *N. gruberi* strains than in *N. lovaniensis* ($P < 0.05$), and finally valine and lysine percentages were higher in *N. gruberi* 1518/1e than in *N. lovaniensis* ($P < 0.05$).

Table 1

Free amino acid composition of three *Naegleria* strains expressed in percentages of the pool

	N. lovaniensis	*N. gruberi*/1f	*N. gruberi*/1e
Aspartic acid	1.39± 1.02	1.60± 0.98	2.17±0.05
Threonine	2.92± 1.13	3.84± 1.18	3.02±0.64
Serine	3.85± 1.06	3.07± 0.65	3.32±1.17
Glutamic acid	4.11± 0.77	3.97± 0.83	3.88±0.85
Proline	27.59±10.09	6.26± 1.21	13.81±3.11
Glycine	5.41± 0.69	4.92± 1.05	5.39±1.20
Alanine	28.30± 7.67	35.33±19.73	28.26±5.88
Cystine*	1.05 —	1.66 —	3.41 —
Valine	2.90± 0.97	5.70± 3.28	4.64±0.82
Methionine	1.11± 0.56	1.15± 0.74	1.16±0.74
Isoleucine	2.25± 1.08	3.39± 2.80	2.79±1.67
Leucine	4.83± 1.89	5.57± 3.24	5.58±1.96
Tyrosine	1.63± 0.79	4.25± 0.62	4.02±0.45
Phenylalanine	3.80± 1.80	6.07± 3.53	6.98±2.63
Tryptophan	Traces	Traces	Traces
Histidine	3.51± 0.70	5.32± 1.32	5.73±0.73
Lysine	3.88± 1.40	6.59± 1.93	6.02±0.83
Arginine	2.09± 1.38	2.11± 2.02	2.01±0.63

*: See text for explanation of SD absence.

Only a few components of the free amino acid pools were at high concentration. Alanine predominated in all strains reaching values above 15 per cent of the pool, proline was also a major component. In contrast,

arginine, aspartic acid and the sulphur amino acids, i.e., cystine and methionine were generally present in small amounts. Tryptophan was detected only in trace amounts.

In several chromatographs, the small cystine peak was so close to that of alanine that both were integrated in the same value, although the contribution of cystine must be very low, for such values were not necessarily higher than when both amino acids were measured separately; therefore, SD for cystine was not presented.

Besides the well characterized amino acids a few small peaks, which remain for the moment undetermined, were observed in the chromatographs.

Protein Amino Acids

After 16 to 72 h hydrolysis the number of nmoles obtained per mg protein presented small variations (from 1500 to 1700 nmol/mg for *N. lovaniensis* and *N. gruberi* 1518/1f, and from 1300 to 1500 nmol/mg for *N. gruberi* 1518/1e). In contrast, after 8 h hydrolysis the values were lower for the three strains (from 900 to 1000 nmol/mg).

A few amino acids reached the highest values after 72 h hydrolysis, as valine and isoleucine (in the three strains), methionine (only in the two strains of *N. gruberi*), phenylalanine (in *N. lovaniensis* and in 1518/1e). Some other amino acids, as serine and tyrosine, showed higher values in the three strains after a short hydrolysis.

To facilitate the comparison of the results, instead of nmol/mg protein, Tables 2-4 show the percentages of each amino acid in relation to the total constitutive amino acids. Percentages of serine, glycine, alanine, tyrosine, histidine and lysine were higher after short hydrolysis, with decreases after prolonged hydrolysis of 20 per cent of serine, 10 per cent of glycine and threonine, from 5 to 20 per cent of alanine (depending on the strain), about 40 per cent of tyrosine, from 10 to 40 per cent of histidine (depending on the strain) and from 25 to 35 per cent of lysine in both *N. gruberi* strains; in contrast there was no destruction of lysine in *N. lovaniensis* after prolonged hydrolysis.

On the other hand, the increase of some amino acids after 72 h hydrolysis in comparison with 8 h hydrolysis ranged from 10 to 66 per cent of valine, from 50 to above 100 per cent of isoleucine and from 10 to 30 per cent of arginine, depending on the strain. Moreover, the highest levels of methionine were also reached after 72 h hydrolysis with increases of 75 and 95 per cent, in comparison with those registered after 16 h hydrolysis, although that holds only for the two *N. gruberi* strains.

Two separate aliquots of the individual dialysates were hydrolyzed and processed under the same conditions to check the confidence of the results. The intervals of variation for each individual amino acid after duplicate assays ranged from 0.0 to 0.6 per cent (mean), 0.15 ± 0.16 per cent standard deviation; the comparison of the data in Tables 2-4 indicates where the variations were significant.

Table 2

Protein amino acid composition of *N. lovaniensis* after five hydrolysis intervals expressed in percentages of total

	8 h	16 h	24 h	48 h	72 h
Aspartic acid	12.87	12.56	13.54	12.34	12.12
Threonine	5.30	4.61	5.05	4.77	4.73
Serine	7.99	7.13	7.72	6.70	6.20
Glutamic acid	12.79	12.89	14.00	12.89	12.67
Proline	Traces	5.59	Traces	5.44	4.91
Glycine	8.25	7.40	7.93	7.16	7.24
Alanine	8.03	7.06	7.59	6.92	7.29
Cystine	1.34	1.17	1.20	1.19	—
Valine	6.70	5.64	5.08	6.01	7.54
Methionine	—	1.15	1.37	1.34	1.11
Isoleucine	3.92	3.87	4.05	5.09	5.88
Leucine	9.08	8.11	8.66	8.15	8.80
Tyrosine	4.61	3.90	3.92	3.32	2.67
Phenylalanine	5.80	5.41	5.86	4.78	5.30
Histidine	3.41	3.10	2.99	3.10	3.07
Lysine	6.57	6.74	7.11	6.94	6.89
Arginine	3.32	3.65	4.46	3.87	3.59

As hydrolyses were conducted in sealed evacuated tubes, the presence of tryptophan should be detected in the chromatographs, at least after short hydrolysis periods. No signs of tryptophan or its decomposition products were perceptible in our chromatographs; consequently no further quantitative determinations of this amino acid were performed.

The percentages recorded for each constitutive amino acid were very similar in the three *Naegleria* strains particularly in both *N. gruberi* strains. The only significant difference recorded between *N. lovaniensis* and *N. gruberi* 1518/1e involved lysine, the percentage of which was lower in the former, particularly after short incubation times in hot HCl. On the other hand, in *N. gruberi* 1518/1f the percentages of lysine, glycine and alanine were higher and that of phenylalanine lower than in *N. lovaniensis*.

Table 3

Protein amino acid composition of *N. gruberi* 1518/1e after five hydrolysis intervals expressed in percentages of total

	8 h	16 h	24 h	48 h	72 h
Aspartic acid	12.06	14.05	11.00	12.48	11.48
Threonine	5.05	5.05	5.37	4.98	4.46
Serine	7.88	8.45	6.98	7.10	6.18
Glutamic acid	12.64	14.87	12.71	13.86	12.81
Proline	Traces	—	5.39	—	6.69
Glycine	8.56	8.58	7.55	8.04	7.73
Alanine	9.15	8.06	7.22	7.36	7.24
Cystine	—	—	0.91	1.44	—
Valine	6.50	5.27	5.68	6.59	7.11
Methionine	Traces	0.96	1.62	1.04	1.69
Isoleucine	3.61	4.18	4.94	5.73	6.27
Leucine	8.37	8.34	8.20	8.36	8.54
Tyrosine	3.69	3.34	3.56	3.52	2.32
Phenylalanine	5.32	4.98	5.07	4.58	5.36
Histidine	4.24	2.12	2.11	3.32	2.33
Lysine	10.08	8.03	8.03	7.90	6.62
Arginine	2.85	3.72	3.65	3.69	3.18

Table 4

Protein amino acid composition of *N. gruberi* 1518/1f after five hydrolysis intervals expressed in percentages of total

	8 h	16 h	24 h	48 h	72 h
Aspartic acid	12.74	12.24	12.20	10.93	12.25
Threonine	5.23	4.69	4.95	5.58	4.81
Serine	8.43	7.81	7.54	6.99	6.88
Glutamic acid	13.02	13.31	13.36	13.02	13.61
Proline	—	5.81	5.26	5.36	—
Glycine	9.17	8.11	8.06	7.88	8.44
Alanine	8.56	7.64	7.59	7.67	8.15
Cystine	Traces	Traces	Traces	—	—
Valine	4.42	5.05	5.68	6.21	7.33
Methionine	—	0.68	1.18	0.43	1.32
Isoleucine	2.95	4.07	4.76	5.39	6.41
Leucine	8.15	8.08	8.20	8.00	8.84
Tyrosine	3.99	3.64	2.98	3.04	2.42
Phenylalanine	5.35	5.16	4.60	4.36	4.99
Histidine	4.78	2.26	2.12	2.98	3.24
Lysine	10.39	7.54	7.25	7.83	7.63
Arginine	2.81	3.91	4.26	4.35	3.66

Discussion

The comparison of both free and constitutive amino acid patterns in the three *Naegleria* strains shows that there are great differences between them. In the free amino acid pools the major constituents are alanine and proline, while within the protein amino acids both dicarboxylic acids, glutamic and aspartic, are by far the most abundant. Significant differences were also found in the percentages of leucine, glycine, serine and threonine. These results are in contrast with those found for mammalian tissues where the composition of the free amino acid pool is generally a reflection of that of the protein amino acids (Meister 1965).

Concerning the free amino acids of the three *Naegleria* strains here studied there are generally considerable variations in the percentages obtained for each one, as shown by the elevated SDs obtained out of three separate experiments. In general, the variability found in the percentages of the free amino acids is much greater than that recorded for the protein amino acids, even taking into account the different times of hydrolysis used for the latter. In spite of that, significant differences in the amount of free proline percentages for each strain were evident; moreover, free valine and lysine values were also different ($P < 0.05$) in *N. lovaniensis* and *N. gruberi* 1518/1e. In other protozoan systems, alanine and proline are also major components, as reported for several *Trypanosoma cruzi* strains (O'Daly et al. 1983), although the bulk of intracellular free amino acids follows a very different pattern. Similarly, free alanine is also a principal component of *Tetrahymena pyriformis* W (Wragg et al. 1965; Alonso and Zubiaur (submitted paper), although in a concentration much lower than in the *Naegleria* strains. In contrast, threonine, glutamic acid and serine concentrations are much higher in the ciliate. There seems to be, therefore, a great diversity in the free amino acid pattern even among the protozoa.

On the other hand, in every analytical study on the amino acids from a protein hydrolyzate it may be questioned whether the values obtained for each one are the true image of the composition of the protein material from which they are derived. During hydrolysis certain labile amino acids are decomposed, and others are difficult to liberate from their peptide bonds. To obviate these drawbacks several aliquots of the same material can be subjected to hydrolysis for different times, and then the analysis of the resulting amino acids is performed.

In the present study, acid hydrolyzates have been analyzed after five hydrolysis intervals looking for the highest value for each amino acid. For several amino acids the maximum value was obtained in the

three strains after the same incubation time in hot HCl (8 h for tyrosine and serine), even similar percentages of destruction were recorded for each amino acid with prolonged hydrolysis. For alanine and histidine the decreases after 72 h hydrolysis varied greatly depending on the strain. When the peptide bond is difficult to break, as it happens with both isoleucine and valine, the highest values obtained were always after 72 h hydrolysis, although the increases in concentration reached by these two amino acids in comparison with short hydrolysis were different for each strain.

Finally, there was a notable variation in the results obtained for lysine; in *N. lovaniensis* the highest percentage was obtained after 24 h hydrolysis, but in both *N. gruberi* strains the maximum was reached after only 8 h of incubation, with substantial decreases after prolonged hydrolysis.

Among the sulphur amino acids, methionine, which usually is rather unstable, proved to be in our assays, quite stable, for the highest levels were attained after 72 h in both *N. gruberi* strains. In contrast, cystine was destroyed after prolonged hydrolysis in all cases. The above mentioned differences may be due to the different disposition of certain amino acids within the protein molecules in each of the strains studied.

When the results obtained here for three *Naegleria* strains are compared with those obtained for *T. pyriformis W* following the same analytical techniques (submitted paper), we find close similarities concerning the protein, or constitutive amino acids; significant differences were detected between the amoebo-flagellates and the ciliate only for proline and, to a lesser degree, for glutamic acid, serine, phenylalanine and threonine. However, we are aware that one must be cautious with this type of comparison as different culture media can influence in some way the amino acid pattern.

REFERENCES

Alonso P. and Zubiaur E. 1985: Phagocytic activity of three *Naegleria* strains in the presence of erythrocytes of various types. J. Protozool., 32, 661-664.

De Jonckheere J. 1977: Use of an axenic medium for differentiation between pathogenic and nonpathogenic *Naegleria fowleri* isolates. Appl. Environ. Microbiol., 33, 751-757.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. 1951: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

Meister A. 1965: Biochemistry of the Amino Acids. Vol. I, Academic Press, New York, London.

O'Daly J. A., Serrano L. E. and Rodriguez M. B. 1983: Free amino acid pool and proteolytic enzymes in *Trypanosoma cruzi* cultured *in vitro*. Int. J. Parasitol., 13, 433-440.

Wragg J. B., Reynolds H. and Pelczar Jr. M. J. 1965: Free amino acids in serine-antagonized cells of *Tetrahymena pyriformis*. J. Bacteriol., 90, 748-754.

Zubiaur E. and Alonso P. 1985: Behaviour of *Naegleria lovaniensis* and *N. gruberi* (1518/1e and 1518/1f) against different lectines. Protistologica, 21, 267-272.

Zubiaur E. and Alonso P. 1987: Relaciones antigénicas entre varias estirpes de *Naegleria*. Microbiología SEM, 3, 25-31.

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Locomotor Response of Five Species of Ciliates to Magnetic and Electrical Stimuli in Varied Chemical Environments

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Synopsis. This research further elucidates the influence of a magnetic field on the locomotion of ciliated protozoa. Five species studied had their movement influenced by the magnetic field. In an attempt to ascertain a possible mechanism for this response, a comparative study was made between it and galvanotaxis. The response of killed protozoa to a magnetic field was also studied. The studies indicated that a magnetic response occurs, but is not brought about by altered ciliary activity.

Numerous organisms, as diverse as eubacteria and vertebrates, have been shown to have the direction of their locomotion affected by magnetic fields. Protists included in this group are *Paramecium caudatum* (Brown 1962, Kogan and Tikhonova 1965) and *Volvox* (Palmer 1963). Most recently, Isquith and Swenson (1987) further elucidated the phenomemon in *P. caudatum*; populations, placed in a high intensity magnetic field, are not uniformly disbursed, but show a significantly greater percentage of organisms at the south than at the north. As soon as the organisms are returned to ambient magnetic conditions, the populations again randomize.

In the current study, in an attempt to further understand magnetic behavior in ciliates, five species were studied, and their chemical environmental conditions were altered. Experiments also were carried out on cells with no ciliary activity to determine whether or not they display any magnetic response. Studies of galvanotaxis were performed so that the two phenomena could be compared and contrasted.

Roberts (1970) suggests that geotaxis might not be an active process, involving stimulus reception and a response of altered ciliary activity. He proposes that a non-uniform density of the cell serves to orient the organism upward or downward and the cilia then propel the organism in the oriented direction. If this is indeed the mechanism for orientation to gravity, Fenchel (1987) does not consider it to be a true taxic response, since it does not involve a ciliary response to a stimulus, as is the case with galvanotaxis. The discovery of magnetite particles in magnetotactic bacteria by Blakemore (1975) helps explain the phenomenon in those organisms and may be relevant to the behavior in *P. caudatum*.

Materials and Methods

Individual protozoa were placed into a channel, 27 mm long × 2 mm wide × 3 mm deep. The channel was positioned parallel to the field produced by a permanent magnet of ram's horn configuration. At the ends of the channel the field strength was 2400 gauss; it decreased to 1600 gauss at the center. Field measurements were done with a gaussmeter (Model Na 505, RFL Industries, Boonton, N. J.). The channel was delineated into three 9 mm regions, designated "north", "middle", and "south", in relation to their proximity to the two poles of the magnet. The entire apparatus was placed onto a Wild stereomicroscope, through which all observations were made. The direction of the magnet as well as the channel were routinely reversed in relation to the other components of the system to eliminate any possible asymmetry.

A single organism was placed into the channel and allowed to resume normal swimming activity. It then was allowed to swim until it had passed from one-third of the channel (i.e., north, middle, or south) to another third, thirty times; this was defined as a single run. Each species was run eighteen times under experimental (magnetic) conditions and an equal number under control conditions. The total time spent in each third of the channel during the eighteen runs was totaled and converted to a percent of the total time for all eighteen runs. The percent time spent in the "north" under experimental conditions was statistically (Student *t* test) compared to the control value for the same end of the channel; the same was done for the "south" end.

Galvanotaxis was measured using a similar chamber, but instead of being placed between the poles of a magnet, copper plates were implanted at its ends. Galvanotactic studies were carried out at 2 amps with 1, 2, 3, 4, 5 or 10 volts.

To ascertain what influence the chemical environment had on the organisms' movements under electric or magnetic influence, runs were done in various fluids. The first were in Cerophyl in which the organisms had been growing. Since the cultures were one week old when used, this medium was significantly altered from its original constitution and was simply designated "growth medium". Other sets of runs were carried out in Chalkley's solution or Chalkley's solution adjusted to pH 5.0, 6.2, 7.5, or 9.5 by addition of concentrated HCl or NaOH, as well as Chalkley's solution with 0.016M $BaCl_2$.

To test whether or not ciliary activity is involved in a magnetic response, cells were fixed with Schaudinn's solution. A cylindrical 20 ml glass funnel, fitted with a 25 cm, 0.45 mμ membrane was placed between the poles of the previously described magnet. The funnel was filled with 15 ml of water; approximately 2 ml of the sample of fixed paramecia was gently added to the top of the liquid. These cells settled into the membrane; the fluid was then drawn through the membrane using gentle suction. The faces of the magnet are 4 cm in diameter; therefore the cells were falling perpendicular to the magnetic field virtually along the entire length of the funnel. Before placing a filter membrane in the funnel it was delineated into two halves by drawing a diameter on it. The membrane was oriented in the funnel so that the diameter was perpendicular to and midway between the two poles. Twenty-six samples were run with the magnet; this was a total of 2489 cells. For one-half the samples the magnet was oriented with the N-pole to the experimenter's right; for the other half, to the left. This was done to remove any assymetry in the system. Twelve control samples were run, containing a total of 1013 cells; the distribution to the left was compared with the distribution to the right. In addition, the experimentals and controls were compared to a theoretical value of a 1 : 1 distibution.

The organisms used were all laboratory cultures, originally obtained from Connecticut Valley Biological Supply Co., Southampton, Mass. Cultures were maintained either in a 0.1% (w/v) solution of bacterized Cerophyl (Cerophyl Laboratories, Inc., Kansas City, Mo.) or bacterized Chalkley's solution.

Results and Discussion

The data in Table 1 indicate that the five species studied (in their undefined growth medium) are attracted to a south magnetic pole; this is indicated by the greater percentage of time spent at the south than

Table 1

Average percentage of time spent in each third of the experimental chamber using growth medium under experimental or control conditions

	Experimental			Control		
	North	Middle	South	North	Middle	South
P. caudatum	25.61 ±5.0	29.09 ±5.2	45.30 ±5.5*	23.98 ±1.4	49.29 ±1.4	26.78 ±2.0
P. multimic.	23.12 ±6.5	32.27 ±9.1	44.61 ±5.9*	24.37 ±0.9	49.73 ±1.5	25.90 ±1.4
P. aurelia	21.67 ±5.1*	39.27 ±12.1	30.03 ±10.4*	25.27 ±1.8	48.46 ±1.8	26.27 ±2.0
P. bursaria	22.16 ±2.6*	45.46 ±2.4	32.37 ±3.3*	25.79 ±1.1	48.34 ±1.6	25.87 ±1.8
Euplotes sp.	20.41 ±4.6*	42.91 ±5.7	36.68 ±4.1*	25.54 ±1.9	48.83 ±1.8	25.60 ±1.3

Each experimental value represents the average of twelve experiments ± standard deviation. Asterisks after an experimental value indicate a significant difference between it and its control value at the 99% level, using the *t* test; significance was only computed for the north and south values.

at the north and by the comparison of the experimental south values with the control values. Comparably there is a statistically significant decrease at the north pole for three of the five species. The phenomenon described here is not like the unidirectional movement to the cathode observed in strong galvanotaxis; in this response the organisms continue to swim back and forth between the north and south poles.

The response to the south is not significantly altered by the chemical environment (see Table 2). Neither altering the pH nor adding $BaCl_2$ to the medium negated the positive response to the south. The galvanotactic response, which has been well studied by numerous investigators was affected by chemical environment. The general trend was for the response (movement to and aggregation at the cathode) to decrease as the pH was increased; $BaCl_2$ caused the organisms to go to the cathode at some voltages and the anode at others (K l e i n e l p, unpublished results). Sensitivity to the environment is what one would expect, since galvanotaxis is a response dependent upon ionic fluxes and ciliary activity.

Table 2

Paramecium caudatum response in different chemical environments

	Experimental			Control		
	North	Middle	South	North	Middle	South
Growth medium	25.61 ±5.0	29.09 ±5.2	45.30 ±5.5*	23.98 ±1.4	49.23 ±1.4	26.78 ±2.0
Chalkey's Soln. Unsupplemented	18.56 ±3.0*	39.39 ±5.7	42.04 ±4.5*	23.73 ±2.8	49.53 ±3.6	25.72 ±2.9
Adjusted to pH 5.0	24.15 ±2.7	32.12 ±3.1	43.72 ±4.0*	26.20 ±4.3	49.55 ±5.3	24.23 ±1.4
Adjusted to pH 6.2	23.19 ±2.6	36.98 ±2.1	39.83 ±3.1*	24.13 ±1.6	47.93 ±1.8	27.93 ±1.2
Adjusted to pH 7.5	21.18 ±2.1*	37.32 ±2.9	41.50 ±2.6*	25.33 ±1.9	48.53 ±0.7	26.12 ±1.4
Adjusted to pH 8.5	21.63 ±2.0*	34.38 ±2.5	43.98 ±3.9*	25.25 ±1.9	48.33 ±1.1	26.40 ±1.9
$BaCl_2$ added	22.95 ±1.4*	41.32 ±2.7	35.68 ±3.2*	26.23 ±0.9	48.23 ±1.3	25.50 ±0.8

Each value represents the average percent of time ± standard deviation spent in the specific third of the chamber under experimental or control conditions. Asterisks after an experimental value indicate a significant difference between it and its control value at the 99% level, using the *t* test; significance was only computed for the north and south values.

When cells fixed with Schaudinn's solution were allowed to fall perpendicular to a magnetic field, a significantly greater number were found at the south (1358 vs. 1131; 54.7% vs. 45.3%) than at the north; the control results were 51.5% vs. 48.5%. Statistically there was a significant difference between the experimental and controls and the experimental and a theoretical 1 : 1 distribution. There was no significant difference between this theoretical value and the controls.

Based upon these experiments and those previously reported, there clearly is a tendency for ciliated protozoa to spend more time at the south pole of an intense magnetic field than at the north pole. The lack of influence of chemical environment on the response and the obtainment of comparable data with fixed cells indicates that this response is not directly due to ciliary activity. The relative constancy of a magnetic influence in altered chemical environments would indicate that it is not based upon a mechanism similar to that for galvanotaxis. This would be the case of the protozoan contained magnetite particles as do magnetotactic bacteria. In the strict sense then perhaps it should not be considered magnetotaxis. To be consistent, however, what is traditionally referred to as geotaxis should also be renamed.

REFERENCES

Blakemore R. P. 1975: Magnetotactic bacteria. Science, 190, 377-379.

Brown F. A., Jr. 1962: Responses of the planarian, *Dugesia* and the protozoan, *Paramecium* to very weak horizontal magnetic fields. Biol. Bull., 123, 264-281.

Fenchel T. 1987: Ecology of *Protozoa*. Sci. Tech. Inc. Madison, Wisconsin, pp. 24-31.

Isquith I. R. and Swenson J. 1987: Magnetic orientation in *Paramecium caudatum*. Acta Protozool., 26, 219-224.

Kogan A. B. and Tikhonova N. A. 1965: Effect of a constant magnetic field on the movement of *Paramecia*. Biofizika, 10, 292-296.

Palmer J. D. 1963: Organismic spatial orientation in very weak magnetic fields. Nature, 198, 1061-1062.

Roberts A. M. 1970: Geotaxis in motile microorganisms. J. Exp. Biol., 53, 687-699.

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Pharmacological Specificity of Beta Blockers-Induced Inhibition of *Paramecium* Phagocytosis

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Synopsis. The effect of β-adrenoceptor antagonists differing in the lipophilicity and membrane stabilizing activity upon *Paramecium* phagocytosis was studied.

The following drugs were examined: propranolol, alprenolol and atenolol. All of them inhibited the process of phagocytosis. The extent of inhibition was dose- and time-dependent being the highest for liposoluble propranolol and alprenolol. Inhibiting effect of the tested drugs was smaller in the starved cells than in the non-starved ones.

Digestive vacuoles formation was completely blocked at 75 μM propranolol and 90 μM alprenolol. At 75 and 90 μM atenolol, a 85% and 88% decrease, respectively, in the number of formed vacuoles was observed. The specific dose- and time-dependent response to atenolol, which is the beta-adrenoceptor antagonist devoid of membrane stabilizing activity, brings evidence about the involvement of beta-receptor in *Paramecium* endocytosis.

Beta adrenoceptor ligands have been found to interact with cell membrane of unicellular protozoan *Paramecium*. In the result of interaction, a modulation of the one of the main cell function — endocytosis may be observed suggesting that both the inhibition (Giordano et al. 1985, Wyroba 1986 a, b, Wyroba 1988 a) and stimulation of this process in *Paramecium* (Wyroba 1987, Wyroba 1988 b) may be under the control of β-adrenergic linked adenylate cyclase system (Wyroba 1987).

Since beta-adrenoceptor antagonists may completely block the process of phagocytosis in *Paramecium* (Giordano et al. 1985, Wyroba 1986 a, Wyroba 1988 b), the specificity of this reaction is of great importance, especially when membrane stabilizing activity of these compounds (Ijzerman et al. 1987, Prichard and Owens 1984)

is taken into account. The aim of present study was to compare the effect of different beta-adrenoceptor antagonists in order to define the extent of specificity in cell response to these drugs. The effect of liposoluble beta blockers — alprenolol and propranolol has been compared with the action of hydrophilic atenolol.

The examined beta blockers differ not only in their liposolubility (Ijzerman et al. 1987) but also in selectivity and membrane-stabilizing activity (Prichard and Owens 1984, Rogers et al. 1986, Ijzerman et al. 1987). Atenolol is about 3 times more potent *in vitro* studies in binding to the beta-1 receptor whereas propranolol appears equally bound to beta-1 and beta-2 receptor similarly to alprenolol (Prichard and Owens 1984). Atenolol is not lipid soluble drug with no membrane stabilizing activity, contrary to propranolol and alprenolol (Prichard and Owens 1984, Ijzerman et al. 1987) and therefore its not yet reported effect on cell function may provide a valuable information concerning the specificity of the response of living cell to beta-adrenoceptor antagonist.

Material and Methods

Paramecium octaurelia (299s) cells growing axenically in medium prepared according to Soldo et al. (1966) were used. Five day-old cultures were collected and washed as described previously (Wyroba 1987). Experiments were performed on the freshly washed cultures (non-starved cells) or on the aseptically starved ciliates. After starvation period (18 h) the cells were washed again with sterile phosphate buffer-salt solution. Two sets of experiments were performed to test the effect of beta-adrenoceptor antagonists on digestive vacuole formation.

(1) Cells were pre-exposed to varying concentrations of l-alprenolol, l-propranolol or atenolol for 20 min, washed twice with a 10-fold volume of buffer and incubated with latex beads for 20 min. Following latex uptake cells were rinsed with buffer solution and fixed with 4% Ca-buffered formalin.

(2) Cells were pulsed with latex beads in the presence of beta blockers.

At various intervals, aliquots of the cells exposed to the three drugs were withdrawn from the incubation media and fixed with 4% Ca-buffered formalin.

The average number of digestive vacuoles in $\geqslant 50$ cells was determined using a dissecting microscope. In all the cases appropriate control was performed and in the experiments described as set 2 it was prepared for each sampling interval.

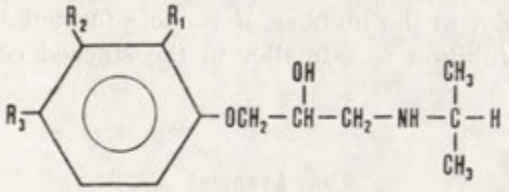
All the procedures were performed at 21-22°C. L-propranolol and atenolol were gifted by Imperial Chemical Industries Ltd. (U.K.), polystyrene latex beads (0.79 μM in diameter) were obtained from Serva (FRG) and l-alprenolol was from Sigma (U.S.A.).

Results

Table 1 demonstrates the chemical structure of the beta-adrenoceptor antagonists tested in this study. All of them exerted a dose- and time-dependent inhibition of digestive vacuole formation in *Paramecium*.

Table 1

Chemical structure of the beta-adrenoceptor blocking drugs investigated in the studies on *Paramecium* phagocytosis



	R_1	R_2	R_3
Alprenolol	$CH_2CH=CH_2$	H	H
Atenolol	H	H	$-CH_2CO-NH_2$
Propranolol	$-CH=CH-CH=CH$		H

When cells were pre-exposed to varying concentrations of the drugs before being pulsed with latex beads, inhibition of phagocytosis started at 10 μM concentration (Fig. 1).

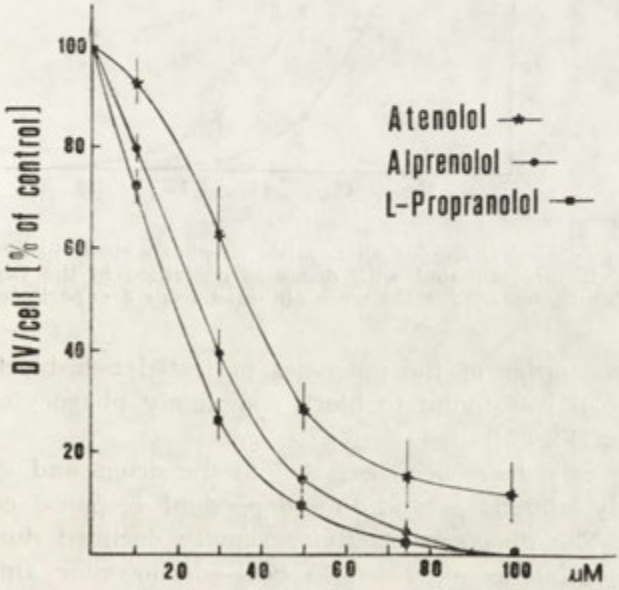


Fig. 1. Effect of propranolol, alprenolol and atenolol on phagocytosis in non-starved *Paramecium* cells. Cells were preincubated for 20 min in the presence of different concentrations of the compounds before addition of latex beads. The average value of digestive vacuoles (DV) formed per cell obtained from at least 50 ciliates is expressed as a percentage of the control. Mean number (± S.D.) of 3 experiments

Drugs behaved according to their liposolubility: the most potent were highly lipophilic propranolol and alprenolol which completely blocked digestive vacuole formation at 75 μM and 90 μM, respectively. Atenolol which is a hydrophilic compound evoked a 85% and 88% decrease, respectively, in the number of vacuole formed at 75 and 100 μM. The extent of inhibition was smaller in the starved cells (Fig. 2), how-

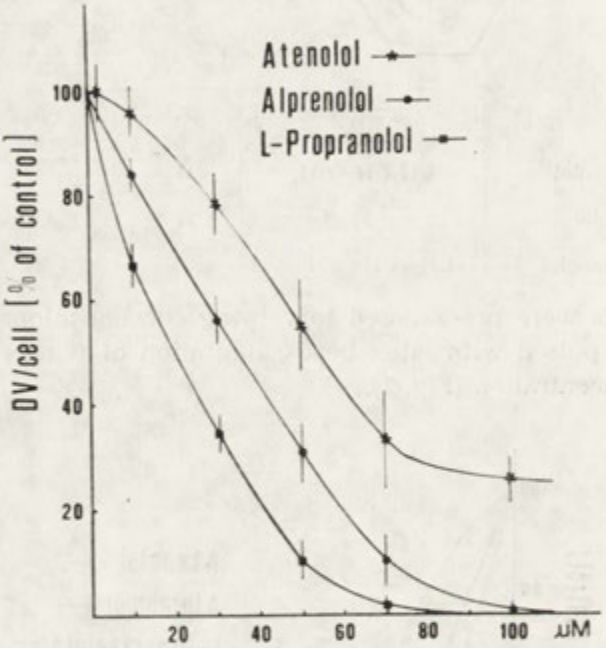


Fig. 2. Effect of beta-adrenoceptor antagonists on phagocytosis in long-term starved *Paramecium*. Cells preincubated with drugs as described in the caption to Fig. 1. Results (mean ± S.D.) were obtained from 3 experiments

ever, the same order of the potencies of tested beta-blockers was observed. Atenolol was found to block completely phagocytosis at higher concentrations (Fig. 3).

When the cells have been exposed to the drugs and latex particles simultaneously, the dose- and time-dependent response can be observed (Fig. 4). The phagocytic activity rapidly declined during the first 20 min of continuous exposure to beta-adrenoceptor antagonists and phagocytic stimulus. When the drugs concentration was 75 μM, the number of digestive vacuoles fell down to 7%, 12% and 30% of the control value, respectively, for propranolol-, alprenolol- and atenolol-exposed cells. Next, a slow recovery of the phagocytic activity was observed start-

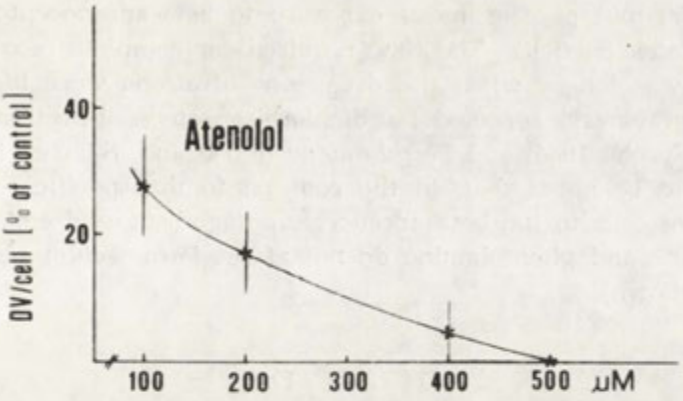


Fig. 3. Effects of concentrations of atenolol on digestive vacuole formation in long-term starved *Paramecium*. Compound tested as described in legend for the Fig. 1. Mean number of 2 experiments

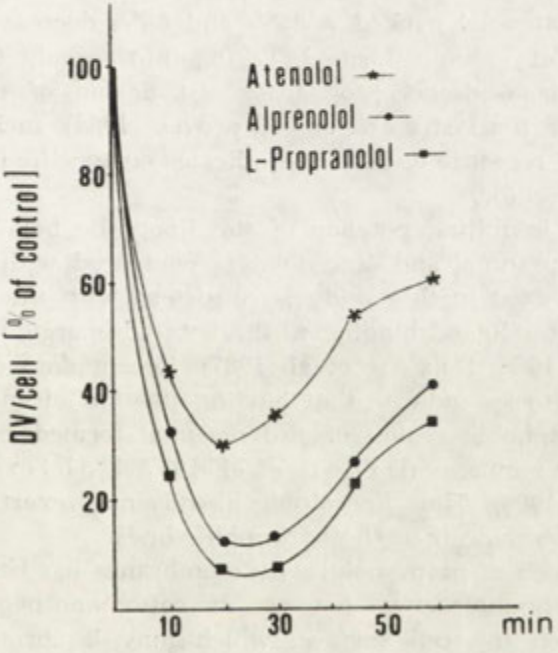


Fig. 4. Effect of simultaneous exposure to propranolol, alprenolol or atenolol and latex beads on digestive vacuole formation in non-starved cells. Drugs concentration was 75 µM. The average value of the digestive vacuoles obtained from $\geqslant 50$ cells was expressed as a percentage of vacuoles formed in the control carried out for each interval in each experiment. Results were obtained from 3 experiments

ing from 30 min of continuous exposure to beta-adrenoceptor antagonists and latex particles. The time required for complete recovery from inhibition was longer when the drug concentrations were higher as it has been previously reported for dichloroisoproterenol-pretreated *Paramecium* (Wyroba 1986) and *Tetrahymena* (Fok and Shockley 1985).

It should be noted that, in the contrast to the specific response of *Paramecium* cells to the beta-adrenergic antagonists α-adrenergic blockers prazosine and phentolamine do not affect *Paramecium* phagocytosis (data not shown).

Discussion

Propranolol, alprenolol and atenolol — β-adrenoceptor antagonists differing in the lipophilicity and membrane stabilizing activity have been found to exert a dose- and time-dependent inhibiting effect on *Paramecium* phagocytosis. The most potent were highly lipophilic drugs propranolol and alprenolol which completely blocked digestive vacuole formation at 75 μM and 100 μM concentrations respectively. At the same concentrations atenolol evoked a 85% and 88% decrease, respectively, in the number of vacuoles formed. Taking into account that atenolol is a hydrophilic beta-adrenoceptor antagonist devoid of membrane stabilizing activity (Prichard and Owens 1984) such a dose- and time-dependent response of the cells indicates on specific interaction with membrane β-receptor.

The higher inhibiting potency of the lipophilic beta-adrenoreceptor antagonists propranolol and alprenolol as compared with the hydrophilic atenolol reported in this study is consistent with the structural features required for ligand binding to the beta-adrenergic receptor (Bar-Sinai et al. 1986, Dixon et al. 1987). Recent data obtained in the different laboratories indicate that binding site for adrenergic ligands is within a hydrophobic region of the receptor formed by a bundle of transmembrane segments (Dixon et al. 1987, Dohlman et al. 1988, Wong et al. 1988). Thus liposoluble ligands may exert a stronger effect on cell membrane than the hydrophilic ones.

The interaction of propranolol with membranes has been found to involve electrostatic interactions between the cation and negatively-charged binding sites on the cell surface, which may be protein, phospholipid or both, in addition to a strong hydrophobic effect which perturbs the arrangement of molecules in the bilayer structure of the membrane (Surewicz 1982, Kubo et al. 1986, Rogers et al. 1986). The effect of propranolol may be explained by its spatial incorporation in the membra-

ne. The hydrophobic aromatic moiety of propranolol partitions into hydrophobic part of lipid bilayer and the charged amine side chain is positioned in the aqueous headgroup region. The three-dimensional structure of propranolol is extended so that the hydrophobic aromatic moiety is well separated from the polar group. Such incorporation of the drug into the membrane may induce a large void ("free volume") at the hydrocarbon lipid core resulting in higher fluidity of the membrane (Govil et al. 1982).

Nosal et al. (1985) studying the interection of beta-adrenoceptor blocking drugs with platelets observed the increase in membrane fluidity as measured with electron spin resonance. The most potent were highly liposoluble drugs alprenolol, exaprolol, metipranolol and propranolol whereas the hydrophilic drugs atenolol and practolol slightly decreased membrane fluidity (Nosal et al. 1985). The mechanism by which atenolol decreases membrane fluidity in platelets or in isolated membranes remains unexplained and the interaction of this drug with the polar membrane part might be of importance (Nosal et al. 1985).

An influence of alprenolol and propranolol has been tested on *Tetrahymena* phagocytosis: Quinones-Maldonado and Renaud (1987) reported that both the drugs had no significant effect on phagocytosis at μM concentrations, but inhibited it at mM concentration. This seems to indicate on a non-specific membrane stabilizing effect of these β-adrenoceptor antagonists on *Tetrahymena*.

The results presented in Fig. 1 and Fig. 2 clearly show that the effects of the tested drugs upon the starved cells are smaller than those observed upon the non-starved ones. The same observation has been reported by Ricketts (1983) who studied the effect of dichloroisoproterenol on *Tetrahymena pyriformis*.

Considering this problem it should be noted that long-term starvation may result in a physiological changement similar to the process of cell ageing. This supposition may be undertaken on the basis of the following observations. The response of the cells differing in the age to the same concentrations of alprenolol is not the same. Non-starved ciliates from the 4-days old culture have been found to be more susceptible to the drug than those from 5-days old culture (Wyroba — unpublished results). When the cells are starved, activation of autophagocytosis and slowing down of heterophagic pathway may be noticed (Fok and Shockley 1985) similarly to the process observed in the aged cells which have fewer food vacuoles than young ones (Smith-Sonneborn and Rodermel 1976).

However, our observations indicate that there is almost no difference between the number or food vacuoles formed by 4-days old and

5-days old cells. Therefore some other explanation of this phenomenon should be considered. One possible reason for the observed smaller susceptibility of both the starved and old cells to beta-adrenoceptor blockers may be related to the changes in lipid composition occurring in *Paramecium* cells upon ageing (Kaneshiro et al. 1979, Rhoads and Kaneshiro 1979). One of the characteristic features of this process is an increase in the ratio of phosphonolipids to total phospholipids (Rhoads and Kaneshiro 1979) associated with a decrease in total amount of lipids per cell.

Phosphonolipids are the stable form of surface membrane lipids and may protect the cell against harmful substances in the environment (Rosenberg 1973). Since a total amount of lipids is smaller in the aged cells and their membranes are enriched in (stable) phosphonolipids it is reasonable to suppose that such ciliates will be less susceptible to beta-adrenoceptor antagonists known to perturbate lipid bilayer than the young cells.

Concluding, it may be stated that inhibition of *Paramecium* phagocytosis induced by beta blockers is not due to their membrane stabilizing activity but it is a concentration-limited and structure-dependent cell response.

As it has been mentioned in Introduction atenolol is a selective beta-blocker without membrane stabilizing activity. Its effect on *Paramecium* phagocytosis is dose- and time-dependent thus indicating on the specific response of the cell to beta-adrenoceptor antagonist. This gives evidence about the involvement of the beta-receptor system in *Paramecium* endocytosis.

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REFERENCES

Bar-Sinai A., Aldouby Y., Chorev M. and Levitzki A. 1986: Association of turkey erythrocyte β-adrenoceptors with specific lipid component. The EMBO Journal, 5, 1175-1180.

Dixon R. A. F., Sigal I. S., Rios Candelore M., Register R. B., Scattergood W., Rands E. and Strader C. D. 1987: Structural features required for ligand binding to the β-adrenergic receptor. The EMBO Journal, 6, 3269-3275.

Dohlman H. G., Caron M. G., Strader C. D., Amlaiky N. and Lefkowitz R. J. 1988: Identification and sequence of a binding site peptide of the β-adrenergic receptor. Biochemistry, 27, 1813-1817.

Ijzerman A. P., Nagesser A. and Garritsen A. 1987: The membrane stabilizing activity of β-adrenoceptor ligands. Quantitative evaluations of the interaction of phenoxypropanolamines with the [^{3}H] batrachotoxinin A 20-α-benzoate binding site on voltage-sensitive sodium channels in rat brain. Biochem. Pharmacol., 36, 4239-4244.

Fok A. K. and Shockley B. U. 1985: Processing of digestive vacuoles in *Tetrahymena* and the effects of dichloroisoproterenol. J. Protozool., 32, 6-9.

Giordano P. A., Wyroba E. and Bottiroli G. 1985: Internalization of cycloheptaamylose-dansyl chloride complex during labelling of surface membrene in living *Paramecium aurelia* cells. Bas. Appl. Histochem., 29, 121-133.

Govil G., Phadke R. S. and Srivastava S. 1982: Magnetic resonance studies on drug-membrane interactions. Current Sci., 51, 493-499.

Kaneshiro E. S., Beischel L. S., Merkel S. J. and Rhoads D. E. 1979: The fatty acid composition of *Paramecium aurelia* cells and cilia: changes with culture age. J. Protozool., 26, 147-158.

Kubo M., Gardner M. F. and Hostetler K. Y. 1986: Binding of propranolol and gentamicin to small unilamellar phospholipid vesicles. Biochem. Pharmac., 35, 3761-3765.

Nosal R., Jancinova V., Ondrias K., Jakubowsky J. and Balgavy F. 1985: The interaction of β-adrenoceptor blocking drugs with platelet aggregation, calcium displacement and fluidization of the membrane. Biochem. Biophys. Acta, 821, 217-228.

Prichard B. N. C. and Owens C. W. I. 1984: Clinical pharmacology of beta-adrenoceptor blocking drugs. In: Beta Blockers in the Treatment of Cardiovascular Disease, (Kostis J. B. and De Felice E. A. eds.) Raven Press, New York, pp. 1-56.

Quinones-Maldonado V. and Renaud F. L. 1987: Effect of biogenic amines on phagocytosis in *Tetrahymena thermophila*. J. Protozool., 34, 435-438.

Rhoads D. E. and Kaneshiro E. S. 1979: Characterization of phospholipids from *Paramecium tetraurelia* cells and cilia. J. Protozool., 26, 329-338.

Ricketts T. R. 1983: Dichloroisoproterenol and digestive vacuolar formation, movement and egestion in *Tetrahymena pyriformis* GL-9. Protoplasma, 115, 25-33.

Rogers J. A., Cheng S. and Betageri G. V. 1986: Association and partitioning of propranolol in model and biological membranes. Biochem. Pharmacol., 35, 2259-2261.

Rosenberg H. 1973: Phosphonolipids. In: Form and Function of Phospholipids (Ansell G. B., Hawthorne J. N. and Dawson R. M. C. eds.). Elsevier Sci. Publ. Co. Amsterdam, 334-344.

Smith-Sonneborn J. and Rodermel S. R. 1976: Loss of endocytic capacity in ageing *Paramecium*. The importance of cytoplasmic organelles. J. Cell Biol., 71, 575-588.

Soldo A. T., Godoy G. A. and van Wagtendonk W. J. 1966: Growth of particle-bearing and particle-free *Paramecium aurelia* in axenic culture. J. Protozool., 13, 492-497.

Surewicz W. K. 1982: Propranolol-induced structural changes in human erythrocyte ghost membranes. A spin label study. Biochem. Pharm. 31, 691-694.

Wong S. K.-F., Slaughter C., Rucho A. E. and Ross E. M. 1988: The catecholamine binding site of the β-adrenergic receptor is formed by juxtaposed membrane spanning domains. J. Biol. Chem., 263, 7925-7928.

Wyroba E. 1986 a: Effect of β-receptor antagonist dichloroisoproterenol on *Paramecium* endocytosis. Acta Protozool., 25, 167-174.

Wyroba 1986 b: Dichloroisoproterenol abolishes endocytosis associated with cell surface staining. Folia Histochem. Cytobiol., 24, 270-271.

Wyroba E. 1987: Stimulation of *Paramecium* phagocytosis by phorbol ester and forskolin. Cell Biol. International Reports, 11, 657-664.
Wyroba E. 1988 a: Endocytosis of cationized ferritin in *Paramecium* cells. Electron Microscopy, EUREM 88, Inst. Phys. Conf., Ser. No. 93, vol. 3, 481-482.
Wyroba E. 1988 b: Involvement of beta-adrenergic receptor system in protozoan endocytosis. Biophysics of membrane transport, abstracts (in press).

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Recovery of *Tetrahymena pyriformis* from the Effects of Colistin. I. A Fluorescence Study

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Synopsis. The behaviour of *Tetrahymena pyriformis* during the recovery from the effects of colistin labelled with dansyl chloride was studied. The findings seem to suggest that in the course of recovery the drug is removed from the cell membrane into the milieu with a simultaneous "blockade" of the structure to new particles of the antibiotic. The likely mechanisms of the process are discussed. The antibiotic used in the study was found to enter in a stable association with the *Tetrahymena* cell membrane.

Physiological adaptation (recovery) is a process which enables some ciliate species to adapt to the continual presence of non-lethal concentrations of cell metabolism inhibitors in the medium. Such response is characteristic of *Tetrahymena* (Frankel 1965, Nelsen 1970, Szablewski 1984, 1985) and *Chilodonella* (Kiersnowska 1982) and consists of the initial inhibition of growth followed by normal development in spite of a continual presence in the medium of the inhibitor which remain active. The response is demonstrated by all cells. In the case of exponentially growing cells, the recovery includes restoration of both the control level of DNA, RNA and protein biosynthesis (Wang and Hooper 1978) and the normal course of morphogenesis (Frankel 1965).

Although physiological adaptation has been subject to numerous studies, the mechanism of the process remains unknown. The results obtained by other authors allow to expect that the process of adaptation will consist of three stages: (1) changes in the cell membrane permeability preventing penetration of more particles of the substance inside the cell (Roberts and Orias 1974); (2) deactivation of the inhibitor in

the cell (Roberts and Orias 1974, Stubblefield 1964); (3) removal of the inhibitor out of the cell (Frankel 1970, Heyer and Frankel 1971).

The antibiotic colistin employed in the studies becomes incorporated into the cell membrane and food vacuole membrane of *Tetrahymena* (Szablewski 1988 a). It has also been established that *Tetrahymena* is capable of recovery in the continual presence of non-lethal concentrations of the antibiotic in the medium (Szablewski 1984, 1985).

If colistin labelled with fluorochrome is added to *Tetrahymena* culture, the antibiotic becomes incorporated into the cell structures with their resulting fluorescence. If after washing out and transfer of the cells to a fresh medium (free of the antibiotic labelled with fluorochrome) loss of fluorescence is observed in the cells after a period of time while naive cells begin to fluoresce after being transferred to the medium in which the experimental cells previously remained one might presume that (1) labelled colistin was removed from the cell membrane during the recovery; (2) the cell membrane has become "blocked" to new particles of the substance although it remains in the medium. The aim of the present study was to verify these hypotheses.

Materials and Methods

The Cells

The experiments were carried out on *Tetrahymena pyriformis*, cultivated at 28°C in Erlenmayer flasks containing 25 ml of the medium. The following media were used: (1) 1.5% proteose-peptone (Difco) + 0.1% yeast extract (Difco) (PPY) and (2) 1.5% proteose-peptone + 0.1% yeast extract + salts (PPYS). The salt-containing medium was prepared according to Plesner et al. (1964).

Preparation of the Complex Colistin-Fluorescent Compound (ColDC)

A fluorescent derivative of colistin was prepared by coupling 1-dimethylaminonaphtalene 5 sulphonyl chloride (dansyl chloride) (Sigma) with the γ amino group of αγ diaminobutyric acid radicals in the polymyxin molecule (ColDC). Colistin was labelled according to the partially modified method originally proposed by Newton 1955 (Szablewski 1988 a).

The Antibiotic

The antibiotic used in the study was manufactured by the Polfa Pharmaceutical Company in Tarchomin. It is a mixture of colistin A (polymyxin E_1) and colistin B (polymyxin E_2). Colistin employed in the study was the sulphate salt. Following the earlier findings (Szablewski 1988 a) the concentration of colistin employed was 0.2 mM, the molecular mass of colistin being 1400.

Microscopical Observations

The samples were examined in a Carl Zeiss Amplival fluorescence microscope. An XBO 50 W lamp was used as a light source. UG 1 (1.5) + BG 12 were employed as excitation filters and OG 4 as a barrier filter. The intensity of fluorescence was determined visually, the following descriptions being used: no fluorescence, weak fluorescence, intense fluorescence.

Experimental Procedure

ColDC at a concentration of 0.2 mM was added to the *Tetrahymena* culture (PPY or PPYS) in an early phase of the exponential growth. After 2 h the cells were centrifuged (700 rotations/min, 10 min) and washed twice in a fresh ColDC-free medium. The centrifuged cells were inoculated into a fresh medium in two Erlenmayer flasks. Prior to the cells' washing out of ColDC and after their inoculation into the fresh medium a sample was taken to investigate the ciliate fluorescence. After inoculation the culture density was ca. 2000 cells/ml. After 24 h the cells from the first Erlenmayer flask were centrifuged. Five ml of naive cells suspension also in the early log phase of cultural growth was added to the medium obtained. After 15 min the cells were inspected for fluorescence. The other Erlenmayer flask was centrifuged after another 24 h (48 h after the inoculation) and the cells were treated in the same manner as the first culture. Fluorescence was also inspected in the experimental cells after 24 and 48 h of cultivation in the fresh medium.

Results

During the cell inoculation into the fresh medium a certain amount of ColDC was transferred into the medium together with the experimental *Tetrahymena*. According to the theoretical calculations ColDC concentration in the fresh medium was less than 4×10^{-3} mM. Such a low concentration of labelled colistin should not produce fluorescence of living cells. A control experiment with naive cells to which colistin at this concentration was added demonstrated that low-intensity fluorescence occurred solely in the cytoplasm of dead cells. In living cells, on the other hand, no organellum revealed fluorescence.

Centrifugation of *Tetrahymena* cells out of ColDC and their transfer into the medium without labelled colistin did not produce immediate loss of fluorescence in the food vacuole membrane or cell membrane (Table 1).

Observations carried out 24 h after transfer of the cells into the fresh medium showed that the cell membrane and food vacuole membrane continued to fluorescence in the experimental *Tetrahymena*, grown either in PPY or PPYS. When the experimental cells were centrifuged and naive cells added to that medium, the latter demonstrated fluorescence of the same structures. Fluorescence was in that case less in-

Table 1

Fluorescence of the cell membrane and food vacuole membrane in *Tetrahymena pyriformis* according to the period of time after washing the cells out of ColDC

Time after washing		15 min before	15 min after	24 h	48 h
Cells	Medium				
Experimental	PPY	++	++	++	−
	PPYS	++	++	++	−
Naive	PPY		×	+	+
	PPYS		×	+	+

++ – intense fluorescence, + – weak fluorescence, − – no fluorescence, × – no fluorescence in the cells in the presence of 4×10^{-3} mM ColDC (for further explanation – see text)

tense than fluorescence of the same structures in the experimental cells (Table 1). In that case, too, the effects were not related to the medium employed. After 48 h the absence of fluorescence was observed in over 80% of the experimental cells. In the remaining cells the intensity of fluorescence was comparable to that observed in naive cells. Similarly to the 24-h study the effects were not related to the type of the medium (Table 1). However, in naive cells added to the medium obtained the membranes were found to fluoresce, the intensity of fluorescence being similar to that seen after 24 h. In that case, too, the final effect was not related to the medium employed.

Discussion

The experiments presented were carried out using colistin labelled with dansyl chloride. However, it seems that the findings may relate to colistin alone. The conclusion is founded upon the earlier findings which demonstrated that *Tetrahymena* "did not distinguish" ColDC from colistin (Szablewski 1988 b).

The present findings seem to indicate a stable incorporation of colistin into the ciliate cell membrane. Both washing the cells out of ColDC and over 24-h long cultivation in a medium without the labelled antibiotic, did not perceptibly influence the intensity of fluorescence in the cell membrane and vacuole membrane of the ciliates. The conclusion confirms the earlier findings (Szablewski 1988 a) as well as observations by other authors (Kuryłowicz 1979).

Two events are likely to account for the loss of fluorescence in the cells found at 48 h after transfer into the fresh medium.

(1) During recovery ColDC was removed out from the cell with a simultaneous "blockade" of the cell membrane against new ColDC particles. (2) In the case of bacteria colistin binds with the liposaccharide fragments of the membranes (Kuryłowicz 1979). It is not known, with what compound(s) colistin binds in *Tetrahymena*. Assuming that lipid components constitute the site, then the loss of fluorescence in the cells may be related to a change in the chemical composition of lipids in *Tetrahymena* as the culture ages. The changes are both quantitative (Hill 1972) and qualitative (Erwin and Bloch 1963, Holtz and Conner 1973). The proces would then consists in "dilution" of ColDC incorporated into the cell membrane during pretreatment, over 48 h after washing the cells out of labelled colistin. If such an event is taken into consideration, it is also necessary to assume that there occurs "blockade" of the cell membrane against ColDC during recovery. It cannot be, however, entirely excluded that colistin is bound by the polysaccharide component of the surface coat. In that case enzymatic removal of the surface coat could lead to the loss of fluorescence of the ColDC complex on the cell surface. Colistin might be bound by both components. More detailed cytochemical studies are required to determine conclusively the compound(s) with which colistin binds.

However, it remains to be established what mechanism is responsible for removal of the toxin out of the cell. In the case of inhibitors penetrating inside the cell, e.g., cycloheximide, the function could be performed by, e.g., contractile vacuoles (Frankel 1970). Heyer and Frankel (1971) suggest that the recovery from inhibitor effects may include induction of the transport sites which pump the drug in the opposite direction. Such sites may have a stable position in the cell or vacuole membrane systems. It is also possible that colistin taken up into the food vacuoles or their membranes is removed out of the cell in the process of exocytosis. How is it possible that the antibiotic permanently incorporated into the cell membrane is removed? It might be achieved in the process of turnover. It has been proved that in this process proteins of the cell surface (Williams 1983) as well as other biomolecules (Watanabe 1971) may undergo exchange. However, the results do not allow unequivocal determination of the mechanism responsible for the removal of colistin out of the *Tetrahymena* cell membrane in the course of recovery.

REFERENCES

Erwin J. A. and Bloch K. 1963: Lipid metabolism of ciliated protozoa. J. Biol. Chem., 238, 1618-1624.

Hill D. L. 1972: The Biochemistry and Physiology of *Tetrahymena*. Cell Biol., Acad. Press Inc., New York and London, 230 pp.

Holz G. G. Jr. and Conner R. L. 1973: The composition, metabolism, and roles of lipids in *Tetrahymena*. In: Biology of *Tetrahymena*, (eds. A. M. Elliott, Dowden, Hutchinson and Ross), Stroudsburgh, 99-122.
Frankel J. 1965: The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. J. Exp. Zool., 159, 113-148.
Frankel J. 1970: An analysis of the recovery of *Tetrahymena* from effects of cycloheximide. J. Cell Physiol., 76, 55-63.
Heyer C. B. and Frankel J. 1971: The kinetics of resensitization of *Tetrahymena* following recovery from effects of cycloheximide. J. Cell Physiol., 78, 411-417.
Kiersnowska M. 1982: Endogenous recovery of cell division in the presence of cycloheximide by the ciliate *Chilodonella cucullulus*: Effects of phenobarbital pretreatment. Experientia, 38, 924-927.
Kuryłowicz W. 1979: Antybiotyki, aktualny stan wiedzy. PZWL, Warszawa, 224 pp.
Nelsen E. M. 1970: Division delays an abnormal oral development produced by colchicine in *Tetrahymena*. J. Exp. Zool., 175, 69-83.
Newton B. A. 1955: A fluorescent derivative of polymyxin: Its preparation and use in studying the site of action of the antibiotic. J. Gen. Microbiol., 12, 226-236.
Plesner P., Rasmussen L. and Zeuthen E. 1964: Technique used in the study of synchronous *Tetrahymena*. In: Synchrony in Cell Division and Growth. (ed. E. Zeuthen). Interscience Publ. New York, London, Sydney, 543-564.
Roberts Ch. T. Jr. and Orias E. 1974: On the mechanism of adaptation to protein synthesis inhibitors by *Tetrahymena*. Facilitation, cross adaptation and resensitization. J. Cell Biol., 62, 707-716.
Stubblefield E. 1964: Cytogenetics of Cell in Culture. (ed. J. C. Harris), 223 pp.
Szablewski L. 1984: The adaptation of *Tetrahymena pyriformis* GL to the continuous presence of colistin in the medium as observed in selected physiological functions. Acta Protozool., 23, 213-235.
Szablewski L. 1985: Adaptation of stomatogenesis and cell division in *Tetrahymena pyriformis* GL to the continuous presence of colistin in the medium. Acta Protozool., 24, 23-35.
Szablewski L. 1988 a: The site of action of colistin on *Tetrahymena pyriformis*. Acta Protozool., 27, 47-54.
Szablewski L. 1988 b: The effect of pretreatment of *Tetrahymena pyriformis* with colistin on the incorporation of this antibiotic into the cell membrane. Acta Protozool., 27, 229-238.
Wang T. Ch. and Hooper A. B. 1978: Adaptation to cycloheximide of macronuclear synthesis in *Tetrahymena*. J. Cell Physiol., 95, 1-12.
Watanabe Y. 1971: Mechanism of synchrony induction. II. Synthesis and turnover of biomolecules in the induction process of synchronous rounding in *Tetrahymena pyriformis*. Exp. Cell Res., 68, 437-441.
Williams N. E. 1983: Surface membrane regeneration in deciliated *Tetrahymena*. J. Cell Sci., 62, 407-417.

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Recovery of *Tetrahymena pyriformis* from the Effects of Colistin. II. A Phagocytosis Study

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Synopsis. The response of *Tetrahymena pyriformis* was studied as seen in the course of recovery after incorporation of colistin labelled with dansyl chloride into the cell membrane. The findings allow to believe that the antibiotic is removed from the cell membrane during recovery. Colistin does not seem to undergo any chemical change within the cell during the physiological adaptation.

The deactivation of a cell metabolism inhibitor and its removal out of the cell are one of the stages of physiological adaptation. As a result *Tetrahymena* cells become insensitive to the permanent presence of a given inhibitor in the medium (Frankel 1965). It is believed that during recovery macromolecules specific for the inhibitor are formed within the cell and deactivate the toxin (Roberts and Orias 1974, Kiersnowska 1982). At the same time the toxin is removed from the cell (Heyer and Frankel 1971).

Colistin is an antibiotic which becomes incorporated into the cell membrane of *Tetrahymena* (Szablewski 1988 a). With the continuous presence of the polymyxin in the medium *Tetrahymena* cells are capable of the physiological adaptation (Szablewski 1984, 1985). Colistin incorporated into the cell membrane is subsequently removed from the cell into the environment after the ciliates have been transferred into a fresh, antibiotic-free medium (Szablewski — in prep.). *Tetrahymena* is known to possess systems which allow it to metabolize either directly or indirectly certain toxins (Fukushima et al. 1977). Other toxins, e.g., some pesticides are accumulated, but not metabolized by *Tetrahymena pyriformis* (Lal et al. 1987). However, it is not known whether during the physiological adaptation colistin undergoes transfor-

mation within the cell. Therapeutic use of colistin includes treatment for bacterial urinary tract infection. Although it is administered orally, colistin reaches a comparatively high concentration in the urine (Korzybski et al. 1977), which may suggest that the antibiotic is not metabolized in the human body.

A phenomenon known as a cross-adaptation is a characteristid feature of physiological adaptation. And thus, the cells adapted to cycloheximide remain sensitive to colchicine and *vice versa* (Frankel 1970) while they develop resistance to antibiotics with a different chemical structure, also acting upon the ribosomes, e.g., cycloheximide and emetine (Roberts and Orias 1974). In the case of inhibitors of close stereochemical affinity, such as cycloheximide and streptomidone, action of one upon the cells produces adaptation to the presence of the other in the medium (Roberts and Orias 1974).

Material and Methods

The Cells

The experiments were carried out on *Tetrahymena pyriformis*. The cells were cultivated at 28°C in Erlenmayer flasks containing 25 ml of the medium. The following media were used: (1) 1.5% proteose-peptone + 0.1% yeast extract (Difco) — PPY and (2) 1.5% proteose-peptone + 0.1% yeast extract + salts (PPYS). The salt-containing medium was prepared according to the method by Plesner et al. (1964).

Preparation of the Complex: Colistin-Fluorescent Compound (ColDC)

Colistin was labelled according to the method proposed by Newton (1955) and partially modified (Szablewski 1988 a). The antibiotic used in the study (the sulphate salt) was manufactured by the Polfa Pharmaceutical Company in Tarchomin. Following the earlier studies (Szablewski 1988 a) ColDC concentration employed in the experiments was 0.2 mM.

The Study of the Course of Phagocytosis

The number of food vacuoles appearing in the cell in the course of absorbing ink suspension (Rotring) diluted with distilled water (1 : 100) was counted. The duration of feeding was established experimentally as 15 min. 0.1 ml of ink suspension was added to cups placed in an incubator and containing 1 ml of cells suspension in the medium. Eventually the cells were fixed with 0.1 ml of 10% neutral formalin and food vacuoles were counted in 100 cells employing the microscope magnification of 320 ×.

Statistical Methods

The results were compared using the "Two way ANOVA with replication" variance analysis method (Sokal and Rohlf 1969). All significance was verified with $p = 0.05$.

The Scheme of Experiments

ColDC at a concentration of 0.2 mM was added to *Tetrahymena* cultures in PPY or PPYS (Culture No. 1 and 2). Culture No. 3 was the control (no ColDC added). After 2 h particular cultures washed twice in PPY or PPYS and transferred into a fresh medium. After 24 h the cells were centrifuged. Cells pretreated for 24 h with 0.2 mM ColDC (Culture A) or naive cells (Culture B) were added to the medium obtained in this way (the medium from Cultures No. 1 and 2 — the experimental medium; the medium from Culture No. 3 — the control medium). Subsequently a phagocytic test was performed. The same procedure was repeated after 48 h. The course of the experiment is presented in detail in Fig. 1.

Results

The findings allow to assume that the phagocytosis rate measured by a number of food vacuoles formed in a period of time depends on many factors. Duration of experiments seems to be in this case the least important as the number of food vacuoles formed in the cells derived from a given culture at 24 h of the experiment does not differ

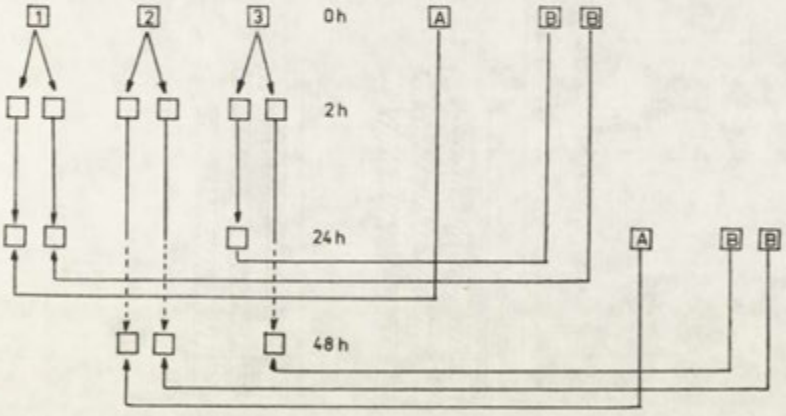

Fig. 1. Scheme of the experiment course. 1 and 2 — the cultures in which the cells were pretreated for 2 h with ColDC, 3 — the control culture (the cells not pretreated with ColDC), A — the cells pretreated for 24 h with ColDC, B — the naive cells

significantly from that at 48 h (Fig. 2). Similarly, differences between particular cultures in the percentage of cells which do not absorb ink suspension are not statistically significant (Fig. 3). On the other hand, the type of medium used, i.e., PPY or PPYS has a greater effect upon the rate of phagocytosis. If naive cells are added to the medium in

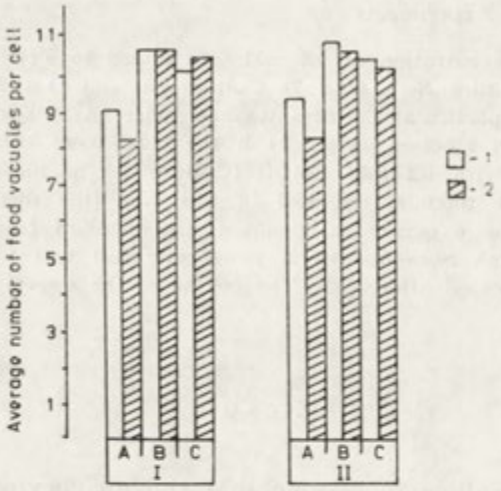

Fig. 2. Average number of food vacuoles per single cell in dependence on the kind of employed medium, treatment of cells and duration of experiment, A — the experimental medium + naive cells, B — the experimental medium + cells pretreated with ColDC, C — the control medium + naive cells, 1 — the culture in PPY, 2 — the culture in PPYS, I — after 24 h of experimentation, II — after 48 h of experimentation

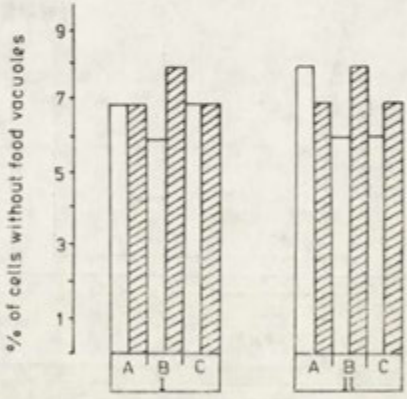

Fig. 3. Percentage of cells not absorbing ink suspension in dependence on the kind of employed medium, treatment of cells and duration of experiment. Symbols like in Fig. 2

which the cells have remained for either 24 or 48 h after pretreatment with ColDC in both cases the phagocytosis rate will be lower in PPYS. The percentage of non-phagocyting cells, however, will be similar. In the remaining cells no effect of the factor was observed upon the mean number of food vacuoles per cell and the percentage of cells which did not absorb ink suspension (Figs. 2 and 3).

The most significant effect was produced by the conditions of the cell culture after their transfer into the experimental medium, i.e., the one obtained by centrifugation the cells previously treated for 2 h with ColDC. If naive cells were added to that medium the number of food vacuoles formed was found to decrease. On the other hand, if the same cells were added to the medium in which *Tetrahymena* not pretreated with ColDC had remained for the same period of time, the phagocytosis rate was higher than that in the previous culture (Diagrams A and C, Fig. 2). When, in turn, the rate of phagocytosis in the cells derived from both cultures was compared to the rate of phagocytosis in the ciliates previously pretreated for 24 h with ColDC and added to the experimental medium (Diagram B, Fig. 2) it was found that:

(1) the rate of the process in naive cells added to the experimental medium differed from the rate of phagocytosis in the two other cultures;

(2) the rate of phagocytosis in naive cells added to the control medium did not differ from the phagocytosis rate in the cells previously treated for 24 h with ColDC and transferred to the experimental medium. In all the cases, however, the percentage of non-phagocyting cells was similar, the differences being not statistically significant (Fig. 3).

Discussion

The process of phagocytosis is very sensitive to any change in the culture conditions (Nilsson 1976, 1979). Accordingly it seems significant that after transfer into the experimental medium naive cells responded with a lowered phagocytosis rate when compared to naive cells transferred to the control medium. Thus it may be expected that the two kinds of medium differ in their physiological action on *Tetrahymena*.

The presence of the phagocytosis inhibitor in the experimental medium might be accounted for the removal of the toxin from the cells into the environment after their pretreatment with ColDC. Another interpretation is also possible, i.e., neither ColDC nor its derivative is the inhibitor of the process. By incorporation into the bacterial cell membrane colistin changes its structure simultaneously increasing its permeability (Sebek 1967, Russel 1977). Thus it exercises a detergent-like action upon bacteria (Korzybski et al. 1977). As a result such compounds as purines, nucleotides, simple monosaccharides and amino acids are released from the cell (Russel 1977). However, when ciliary reversal (Brutkowska et al. 1974, Dryl 1974) was studied

in *Tetrahymena* as effected by potassium ions in the presence of colistin such action of the antibiotic was not confirmed (Szablewski 1984). Still it seems that the release of various compounds from *Tetrahymena* cells due to the antibiotic cannot be entirely excluded. On the other hand, the compounds which constitute inherent and extremely important components of the cell are not very likely to inhibit the process of phagocytosis to such degree. More probably, ColDC or its derivative removed from the cell during recovery is the inhibitor. Is ColDC metabolized in the cell during the physiological adaptation? The answer to the question is related to the phenomenon known as cross-adaptation (Roberts and Orias 1974), discussed in a greater detail in the introduction. Cells previously treated with ColDC and thus adapted to the inhibitor, when transferred into the experimental medium demonstrated the phagocytosis rate similar to that seen in naive cells transferred into the control medium, i.e., they did not respond to the presence of the inhibitor in the medium. Accordingly, colistin might be expected not to undergo chemical changes inside the cell during the physiological adaptation or if such change occurs its products either act on the cell membrane similarly to ColDC or demonstrate a marked stereochemical affinity to ColDC. Decomposition of ColDC into colistin and dansyl chloride can be excluded in this case. Dansyl chloride at this concentration would produce fluorescence of the cell cytoplasm. Similar studies employing fluorescence methods demonstrated incorporation of the substance removed from the cell membrane during recovery exclusively into the cell membrane (Szablewski 1989).

How to explain then the findings that naive cells transferred into the experimental medium demonstrated different phagocytosis rates according to the medium (PPY or PPYS)? The dynamics of cell multiplication is higher in PPYS than in PPY (Szablewski — unpublished data). As a result the rate of ColDC removal from the cell membrane of *Tetrahymena* previously treated with ColDC and transferred into a fresh, antibiotic-free medium will differ in the two cases. Thus ColDC concentration in the experimental medium PPYS might be expected to be higher than in the experimental medium PPY. This, however, is a hypothesis which the present findings cannot conclusively prove. More precise experiments in the area of biochemistry and analytical chemistry would be necessary.

Why in the case of phagocytosis are observed the effects of *Tetrahymena* pretreatment with colistin while such effects are not seen in the case of albumin uptake (Szablewski 1989)? The reason may be different. The fact may be due, e.g., to different sensitivity of the two processes to the polymyxin employed in the study. Other findings

suggest that the relationship between the inhibitor concentration during pretreatment and its level on subsequent addition to the culture (Szablewski 1988 b, Szablewski — in prep.).

REFERENCES

Brutkowska M., Dryl S. and Mehr K. 1974: Action of detergents on motility of *Tetrahymena pyriformis* GL. J. Protozool., 21, 440-441.

Dryl S. 1974: Behavior and motor response of *Paramecium*. In: *Paramecium*. A Current Survey. (Ed. W. J. Van Wagtendonk), Elsevier Sci. Publ. Company, Amsterdam, London, New York, 165-218.

Frankel J. 1965: The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. J. Exp. Zool., 159, 113-148.

Frankel J. 1970: An analysis of the recovery of *Tetrahymena* from effects of cycloheximide. J. Cell Physiol., 76, 55-64.

Fukushima H., Nagao S., Okano Y. and Nozawa Y. 1977: Palmitoyl-coenzyme a desaturase, a possible key enzyme for temperature adaptation in *Tetrahymena* microsomes. Biochem. Biophys. Acta, 488, 442-453.

Heyer C. B. and Frankel J. 1971: The kinetics of resensitization of *Tetrahymena* following recovery from effects of cycloheximide. J. Cell Physiol., 78, 411-417.

Kiersnowska M. 1982: Endogenous recovery of cell division in the presence of cycloheximide by the ciliate *Chilodonella cucullulus*: Effects of phenobarbital pretreatment. Experientia, 38, 924-927.

Korzybski T., Kowszyk-Gindifer Z. and Kuryłowicz W. 1977: Antybiotyki, pochodzenie, rodzaje i właściwości. T. II, PZWL, Warszawa 1771 pp.

Lal S., Saxena D. M. and Lal R. 1987: Uptake, metabolism and effects of DDT, fenitrothion and chlorpyrifos on *Tetrahymena pyriformis*. Pestic. Sci., 21, 181-191.

Newton B. A. 1955: A fluorescent derivative of polymyxin: its preparation and use in studying the site of action of the antibiotic. J. Gen. Microbiol., 12, 226-236.

Nilsson J. R. 1976: Physiological and structural studies on *Tetrahymena pyriformis* GL. C. R. Trav. Lab. Carlsberg, 40, 217-355.

Nilsson J. R. 1979: Phagotrophy in *Tetrahymena*. In: Biochemistry and Physiology of *Protozoa*. Sec. ed., 2, 339-379.

Plesner P., Rasmussen L. and Zeuthen E. 1964: Technique used in the study of synchronous *Tetrahymena*. In: Synchrony in Cell Division and Growth. (Ed. E. Zeuthen), Intersci. Publ., New York, London, Sydney, 543-564.

Roberts Ch. T. Jr. and Orias E. 1974: On the mechanism of adaptation to protein synthesis inhibitors by *Tetrahymena*. Facilitation, cross adaptation and resensitization. J. Cell Biol., 62, 707-716.

Russel S. 1977: Antybiotyki. PWN, Warszawa, 323 pp.

Sebek O. K. 1967: Polymyxins and circulin. In: Antibiotics, vol. I: Mechanism of Action. (Eds. Gottlieb D. and Shaw P. D.), Springer Verlag, Berlin.

Sokal R. R. and Rohlf F. J. 1969: Biometry. W. H. Freeman and Co., San Francisco, 776 pp.

Szablewski L. 1984: The adaptation of *Tetrahymena* pyriformis GL to the continuous presence of colistin in the medium as observed in selected physiological functions. Acta Protozool., 23, 213-235.

Szablewski L. 1985: Adaptation of stomatogenesis and cell division in *Tetrahymena pyriformis* GL to the continuous presence of colistin in the medium. Acta Protozool., 24, 23-35.

Szablewski L. 1988 a: The site of action of colistin on *Tetrahymena pyriformis*. Acta Protozool., 27, 47-54.
Szablewski L. 1988 b: The effect of pretreatment of *Tetrahymena pyriformis* with colistin on the incorporation of this antibiotic into the cell membrane. Acta Protozool., 27, 229-238.

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The Effect of Pretreatment with Colistin and Cycloheximide on the Albumin Uptake in *Tetrahymena pyriformis*

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Synopsis. The pretreatment of *Tetrahymena* with colistin and cycloheximide did not affect the albumin uptake by the ciliates during their subsequent exposure to the drugs. The phenomenon known as cross-adaptation was not observed. However, the cultivation of *Tetrahymena* in a particle-free medium proved to decrease considerably the ciliates' resistance to colistin at the concentrations used in the study (2.25 mg/ml) as compared to the cell cultivated in control medium allowing formation of food vacuoles.

Tetrahymena pyriformis is a ciliate which ingest food substances from the medium by formation of membrane-limited vacuoles (endocytosis). Cells grown in nutritive media are capable of producing digestive vacuoles which can be observed with the light microscope (Rasmussen 1976, Ricketts 1973). It is, however, possible that the endocytic uptake of external materials might also occur by a micropinocytic mechanism (Elliott and Clemons 1966) or without membrane invagination (Rasmussen 1976). Thus it may be expected that these mechanisms are also responsible for penetration of various inhibitors into the cell.

A decreased sensitivity of the ciliates to subsequent exposure to the toxin may be observed on addition of cell metabolism inhibitors to a *Tetrahymena pyriformis* culture (Roberts and Orias 1974). The effect is associated with a process known as physiological adaptation and seen with both inhibitors which enter the cell (Frankel 1965, 1969) and those which do not penetrate inside the cell (Szablewski 1984, 1985). The mechanisms of physiological adaptation are thought to include a change in the permeability of the cell membrane allowing

penetration of more toxin particles inside the cell (Roberts and Orias 1974).

A question therefore arises whether the pretreatment of *Tetrahymena* with the investigated inhibitors followed by addition of the same inhibitor to the culture will produce an alteration of the cell membrane permeability? Will a phenomenon known as cross-adaptation (Roberts and Orias 1974) occur although the inhibitors employed in the studies differ in the mode and site of action?

To answer these questions cycloheximide (CHX) and colistin were used in the present study. Cycloheximide, an inhibitor of protein synthesis, is an antibiotic which can inhibit food vacuole formation and micropinocytosis (Ricketts and Rappitt 1975). Colistin has been found to form a stable connection with the cell membrane of *Tetrahymena* (Szablewski 1988 a) and inhibit the process of phagocytosis (Szablewski 1984).

Material and Methods

The Cells

The experiments were carried out on an amicronucleate strain of *Tetrahymena pyriformis* GL. The cultures were maintained in 25-ml test-tubes containing 5 ml of the medium (1.5% proteose-peptone + 0.1% yeast extract — Difco) at 28°C. In order to decrease the rate of food vacuole formation in the process of phagocytosis the experiments were conducted using 1.5% proteose-peptone + 0.1% yeast extract, sterile filtered through a Milipore membrane (pore size = 0.22 μm) as a particle-free medium (Rasmussen 1973).

0.5 ml of cells from the nutrient medium (containing particles, without previous filtration through a Milipore membrane) was inoculated into a test-tube containing 5 ml of the particle-free medium. The test-tubes were kept slanted at a temperature of 28°C. After next 24 h the cells were inoculated again in the same manner and CHX or colistin at appropriate concentrations were added to particular cultures. After 24-h pretreatment with the inhibitors CHX or colistin at concentrations of 0.7 μg/ml and 2.25 mg/ml respectively were added to the cultures, followed after 15 min by labelled human albumin (see Table 1) at a concentration of 0.1 mM. Albumin at so low concentration only slightly stimulate the formation of food vacuoles (Ricketts and Rappitt 1975).

Chemicals

The minimum antibiotic dose used for the purpose of pretreatment and added after the pretreatment was determined as ca. 10% of the concentration producing complete inhibition of the culture growth, calculated as 0.7 μg/ml in the case of CHX (Frankel 1969) and 2.25 mg/ml for colistin (Szablewski 1981).

Stock solutions were prepared with sterile water distilled three times and used immediately. CHX used in the study was manufactured by the Nutritional

Biochemicals Corporation, Ohio and colistin by the Polish Pharmaceutical Company "Polfa".

The human albumin (Biomed) was labelled with dansyl chloride (Sigma) using the method originally proposed by Weber (1952).

Microscopical Observations

The samples were examined in a Carl Zeiss Amplival fluorescence microscope. XBO 50 W lamp was used as a light source. UG 1 (1.5) + BG 12 were employed as excitation filters and OG 4 as a barrier filter. The observations were conducted at 15 min after addition of the labelled albumin. The intensity of fluorescence was determined visually, the following descriptions being used: no fluorescence, weak fluorescence, evident fluorescence.

Results

Over the 24-h period of *Tetrahymena* cultivation in the particle-free medium the culture density increased by ca. 60% (Szablewski — unpublished data), thus demonstrating a markedly longer time

Table 1

The effect of pretreatment of *Tetrahymena pyriformis* with various concentrations of colistin and cycloheximide on the labelled albumin uptake. Concentrations of colistin and CHX during pretreatment expressed in mg/ml and μg/ml respectively

No. of culture	Pretreatment with	Added after 24 h	Results
1	———	albumin	++
2	———	colistin 2.25 mg/ml + albumin	+
3	———	CHX 0.7 μg/ml + albumin	+
4	colistin 2.25	colistin 2.25 mg/ml + albumin	+
5	colistin 4.50	colistin 2.25 mg/ml + albumin	+
6	colistin 9.00	colistin 2.25 mg/ml + albumin	—
7	colistin 18.00	colistin 2.25 mg/ml + albumin	—
8	CHX 0.7	CHX 0.7 μg/ml + albumin	+
9	CHX 1.4	CHX 0.7 μg/ml + albumin	+
10	CHX 2.8	CHX 0.7 μg/ml + albumin	+
11	CHX 5.6	CHX 0.7 μg/ml + albumin	+
12	colistin 2.25	CHX 0.7 μg/ml + albumin	+
13	colistin 4.50	CHX 0.7 μg/ml + albumin	—
14	colistin 9.00	CHX 0.7 μg/ml + albumin	—
15	colistin 18.00	CXH 0.7 μg/ml + albumin	—
16	CHX 0.7	colistin 2.25 mg/ml + albumin	+
17	CHX 1.4	colistin 2.25 mg/ml + albumin	+
18	CHX 2.8	colistin 2.25 mg/ml + albumin	+
19	CHX 5.6	colistin 2.25 mg/ml + albumin	+

++ — evident fluorescence of the cytoplasm in all cells, + — very weak fluorescence of the cytoplasm in 80% of cells, no detectable fluorescence in the remaining cells, — — lethal dose for about 100% of cells.

of cell generation. The effect was due to a considerably lower rate of food vacuole formation in the cells (Rasmussen 1973). On addition of the labelled albumin evident fluorescence of the cell cytoplasm was observed in the control culture (Culture No. 1, see Table 1). After a longer exposure of the ciliates to the albumin (ca. 30 min) single phagocytic vacuoles could be seen in some cells.

Addition to the naive cells of CHX at 0.7 μg/ml or colistin at 2.25 mg/ml followed by the labelled albumin produced an evident decrease in the *Tetrahymena* cytoplasm fluorescence as compared to the control (Cultures No. 2 and 3, Table 1). That suggested a decrease in the labelled albumin uptake by *Tetrahymena* effected by the antibiotics.

The pretreatment of the cells with 2.25 mg/ml and 4.5 mg/ml of colistin followed by the antibiotic at 2.25 mg/ml did not seem to change the intensity of cell cytoplasm fluorescence as compared to the cells which did not receive pretreatment, being exposed to the antibiotic for the first time (Cultures No. 4 and 5, Table 1). On the other hand, higher concentrations of colistin used for pretreatment produced death in nearly 100% of the cells (Cultures No. 6 and 7, Table 1).

A 24-h exposure of the ciliates to colistin at the concentration of 2.25 mg/ml followed by addition of 0.7 μg/ml CHX also caused an evident decrease in the intensity of cell cytoplasm fluorescence as compared to the control (Culture No. 12, Table 1). Higher concentrations of colistin, however, caused in that case lethal effects in nearly 100% of the cells (Cultures No. 13, 14, and 15, Table 1).

The pretreatment of *Tetrahymena* with CHX at different concentrations, followed by addition to the cultures of CHX at a concentrations of 0.7 μg/ml or colistin at 2.25 mg/ml similarly inhibited the albumin uptake in *Tetrahymena* observed as a decreased intensity of cell cytoplasm fluorescence as compared to the control. In this case, however, contrary to the pretreatment with colistin no lethal effects were seen in any of the cultures (Cultures No. 8 to 11, and 16 to 19, Table 1).

Discussion

The present findings suggest that *Tetrahymena* may absorb the labelled albumin without forming food vacuoles. However, due to the lack of electron microscopy studies the conclusive determination of the mechanism of labelled albumin uptake by *Tetrahymena* is not possible. The uptake can occur across the plasma membrane, by micropinocytosis or by pinocytosis (Rasmussen 1976).

The pretreatment of *Tetrahymena* with the two inhibitors under study did not produce any noticeable changes in the permeability of the cell membrane to the labelled albumin. It was found that the preincubation of cells in concentrations of CHX lower than those that cause a detectable lag in growth or a decrease in the growth rate (< 0.01 μg/ml) significantly reduced the growth lag exhibited on subsequent exposure to higher cycloheximide concentrations. This phenomenon is termed "facilitation" (Roberts and Orias 1974). It is likely that the phenomenon of "facilitation" does not concern all the physiological functions of *Tetrahymena*. The findings do not provide the conclusive answer. However, the comparison of the present findings with the results concerning the effect of colistin pretreatment upon the incorporation of the antibiotic into the cell membrane demonstrate different sensitivity of the cells to the employed colistin concentrations. In order to prevent the incorporation of colistin labelled with dansyl chloride into the *Tetrahymena* cell membrane it was necessary to employ pretreatment of the ciliates with 16 mM of the antibiotic (Szablewski 1988 b). Different media may account for differences in the cell sensitivity to the antibiotic presence in the milieu. Since a particle-free medium was employed to investigate the albumin uptake practically no phagocytosis occurred which might have indirectly produced increased sensitivity of the cells to colistin. Because a nutrient medium was used in the second experiment the lethal dose of the antibiotic was much higher. Another explanation of the phenomenon is also likely. Colistin might be adsorbed on the suspension particles with the result that in their presence active colistin concentration in the solution might be much lower than in the particle-free medium.

The present findings seem to suggest that in this case there is no phenomenon of the so-called "cross-adaptation" (Roberts and Orias 1974). This result is in agreement with the expectations since according to the authors cross-adaptation occurs if the sites of action of the inhibitors in the cell are either identical or stereochemically similar while in the case of colistin and CHX none of these is true.

Observations by various authors indicate that the mechanism of phagocytosis in *Tetrahymena* differs from the uptake of chemicals by means of pinocytosis or across the cell membrane (Nilsson 1979, Rasmussen 1976). Similarly, if the processes are considered in terms of physiological adaptation, significant differences can be also found. Thus the rate of phagocytosis (measured by the number of food vacuoles formed in a given period of time) in *Tetrahymena* following colistin addition to the culture depends on the previous exposure of the ciliates to the antibiotic. Besides, the pretreatment of cells with colistin lowers the sensi-

tivity of *Tetrahymena* to colistin labelled with dansyl chloride, i.e., a phenomenon analogous to "cross-adaptation" is observed (Szablewski 1988 b). These observations may suggest that a change in the permeability of the *Tetrahymena* cell membrane effected by the inhibitor is a non-specific mechanism in the process of physiological adaptation. In this case non-specificity means that each exposure of the cells to the inhibitor produces a decrease (possibly of a similar degree) in the permeability of the cell membrane while a pretreatment with this inhibitor does not seem to affect the permeability of the membrane to albumin.

REFERENCES

Elliott A. M. and Clemons G. L. 1966: An ultrastructural study of ingestion and digestion in *Tetrahymena pyriformis*. J. Protozool., 13, 311-323.

Frankel J. 1965: The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. J. Exp. Zool., 159, 113-148.

Frankel J. 1969: The relationship of protein synthesis to cell division and oral development in synchronized *Tetrahymena pyriformis* GL-C: An analysis employing cycloheximide. J. Cell Physiol., 74, 135-148.

Nilsson J. R. 1979: Phagotrophy in *Tetrahymena*. In: Biochemistry and Physiology of *Protozoa*, sec. ed., vol. 2, Academic Press, Inc., 339-379.

Rasmussen L. 1973: On the role of food vacuole formation in the uptake of dissolved nutrients by *Tetrahymena*. Exp. Cell Res., 82, 192-196.

Rasmussen L. 1976: Nutrient uptake in *Tetrahymena pyriformis*. Carlsberg Res. Commun., 41, 143-167.

Ricketts T. R. 1973: The relationship of endocytosis and digestive enzymes in *Tetrahymena*. In: Progress in Protozoology, Abstr. IV Int. Congr. Protozool., p. 344.

Ricketts T. R. and Rappitt A. F. 1975: A radioisotopic and morphological study of the uptake of materials into food vacuoles by *Tetrahymena pyriformis* GL-9. Protoplasma, 86, 321-337.

Roberts Ch. T. Jr. and Orias E. 1974: On the mechanism of adaptation to protein synthesis inhibitors by *Tetrahymena*. Facilitation, cross adaptation and resensitization. J. Cell Biol., 62, 707-716.

Szablewski L. 1981: The effect of selected antibiotics on *Tetrahymena pyriformis* GL. Acta Protozool., 20, 309-322.

Szablewski L. 1984: The adaptation of *Tetrahymena pyriformis* GL to the continuous presence of colistin in the medium as observed in selected physiological functions. Acta Protozool., 23, 213-235.

Szablewski L. 1985: Adaptation of stomatogenesis and cell division in *Tetrahymena pyriformis* GL to the continuous presence of colistin in the medium. Acta Protozool., 24, 23-35.

Szablewski L. 1988 a: The site of action of colistin on *Tetrahymena pyriformis*. Acta Protozool., 27, 47-54.

Szablewski L. 1988 b: The effect of pretreatment of *Tetrahymena pyriformis* with colistin on the incorporation of this antibiotic into the cell membrane. Acta Protozool., 27, 229-238.

Weber G. 1952: Polarization of the fluorescence of macromolecules. 2. Fluorescent conjugates of ovalbumin and bovine serum albumin. J. Biochem., 51, 155-167.

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Effects on Uptake by and Metabolism of Aldrin and Phorate in a Protozoan, *Tetrahymena pyriformis*

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Synopsis. Growth of *Tetrahymena pyriformis* was retarded by aldrin (10, 20 and 50 ppm) and phorate (1, 10 and 20 ppm) but the organism showed a tendency to recover from the toxic effects of these insecticides with time. At 100 ppm of aldrin and 50 ppm of phorate, the organisms lysed within 6 h of treatment. The organism picked up 32.1 to 924.3 ppm of aldrin and 21.1 to 948.1 ppm of phorate from culture media containing 0.1, 0.5 and 1.0 ppm of aldrin and 2.5, 5 and 10 ppm of phorate respectively. Uptake and bioconcentration by the organism of aldrin and phorate has been inversely dependent on their water solubility. *Tetrahymena pyriformis* metabolized aldrin to dieldrin while phorate was unchanged by the organism.

Extensive use of insecticides for agricultural and health programmes has created serious concern over the years because of their toxic effects on non-target organisms. In the aquatic environment, the ciliate protozoans are very important as they constitute a major group in the ecosystem (Butler 1977, Williams 1977) and are efficient nutrient regenerators (Johannes 1968). As these protozoans graze on algae and bacteria, any harmful effect of the insecticides on the ciliates may indirectly affect the algal and bacterial populations also.

Further, microorganisms act on the insecticides by rapidly accumulating, metabolizing and/or transporting these chemicals through successive trophic levels in food chains. Uptake and metabolism of insecticides by various groups of algae, fungi and bacteria have been extensively investigated (Lal and Saxena 1982, Lal 1984, Lal et al. 1987 a) but there is lack of information on the interaction of insecticides with protozoans. The present investigation has been aimed at the effects on,

uptake by and metabolism of aldrin and phorate in a ciliate protozoan, *Tetrahymena pyriformis*.

Material and Methods

Stock culture of *Tetrahymena pyriformis* (Syngen-1) was obtained from Dr. J. G. Jones, Department of Biochemistry, University of Hull, U.K., and was maintained axenically at 27±1°C in 15 ml centrifuge tubes containing 2 ml of 1% proteose peptone (Difco) supplemented with 5% sodium chloride and 0.3% yeast extract (Difco).

Aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydroexo-1,4-endo-5,8-dimethanonaphthalene), dieldrin (1,2,3,4,10,10-hexachloro-6,7,-epoxy-1,4,4a,5,6,7,8,8a-octahydro--exo-1,4-endo-5,8-dimethanonaphthalene), and phorate (0,0-diethyl S-(ethylthiomethyl) phosphorodithioate) were obtained from Dr. E. Thompson, U.S. Environmental Protection Agency, Research Triangle Park, NC, U.S.A. The purity of the insecticides was always above 94.5%. Fresh stock solutions of insecticides were made in acetone.

For growth studies, 5 ml of *Tetrahymena* culture was raised in 15 ml test tubes by transferring one loop of 72 h old culture. After allowing to grow for 24 h, the cultures in triplicate were treated with 10, 20, 50 and 100 ppm of aldrin and 1, 10, 20 and 50 ppm of phorate dissolved in acetone. Acetone concentration up to 0.1% was not toxic to the organisms (Table 1) and in all experiments the solvent concentration was always 0.1% or less. The number of organisms was counted every day for 5 days with an haemocytometer. Accordingly the samples were fixed with an equal volume of neutral formalin.

Table 1

Effect of Acetone (0.1%) on growth of *Tetrahymena pyriformis*

Exposure (Days)	Number of organisms/ml × 10^4	
	Without acetone	With acetone
1	4.20±0.62	4.14±0.28
2	6.82±0.56	6.90±0.85
3	14.36±0.21	14.30±0.76
4	16.32±0.25	16.14±0.40
5	15.77±0.50	15.87±0.48

For uptake and metabolism studies, 3 ml of proteose peptone medium in centrifuge tubes was inoculated with *Tetryhamena* by transferring one loop from 72 h old culture and allowing to grow for 24 h. Two ml of fresh medium was added to these cultures and treated with 0.1, 0.5 and 1 ppm of aldrin or 2.5, 5 and 10 ppm of phorate. All treatments were done in triplicates. Controls containing only insecticides and culture medium, culture medium and organisms and culture medium alone were also run under similar conditions. After 2, 4, 6, 8, 10 and 12 h of exposure, *T. pyriformis* was separated from the culture medium by centrifuging at 6000 rpm for 10 min. The pellet was washed three

times with insecticide-free distilled water to remove the insecticides adsorbed to the organisms. The insecticide and its metabolites were extracted with acetone, the residue was dissolved in hexane and subjected to gas liquid chromatography (GLC). The uptake of insecticides was expressed in $\mu g\ g^{-1}$ dry weight. Corresponding cultures in triplicates were pelleted, transferred to aluminium cups and dried to constant weight at 80°C for 12 h. Bioconcentration factor was obtained by dividing the concentration of aldrin or phorate picked up the organism by the concentration of respective insecticide initially added to the medium (L a l et al. 1987 a).

Qualitative and quantitative analyses of insecticides were carried out in a Packard 438 GLC fitted with a Shimadzu C-R2A integrator. Electron caputer detector (ECD) and a glass column (2 m, length; 2 mm, internal diameter) packed with 4% SE 30 (Trifluoromethyl silicone) + 6% OV 210 (Trifluoropropylmethyl silicone) was used for the analysis of aldrin while a flame photometric detector (FPD) and and a glass column (2 m, length; 2 mm, internal diameter) packed with 1.5% OV 17 (methyl silicone, 50% phenyl substituted) + 1.95% OV 202 (Trifluoropropyl silicone) was used for the analysis of phorate. The operating temperatures of column, detector and injector were 200, 220 and 220°C, respectiely. Flow rates of N_2, H_2 and air were 15, 105 and 145 ml/min respectively. Recovery of the insecticides from organisms and culture was 82.0 to 104.8%.

Results and Discussion

Aldrin and phorate showed dose dependent inhibition of growth in *T. pyriformis* (Fig. 1 and 2). The effect of insecticides was more severe initially and the organisms recovered from the effect with the passage of time. For instance, 1 day after treatment with 10, 20 and 50 ppm of aldrin, the population was reduced by 21.3 to 62.1% of control whilst the range of inhibition was 4.3 to 42.9% of control after 5 days. Phorate was more toxic to *T. pyriformis* and complete mortality of the organism was noticed at 50 ppm within 6 h of treatment. At lower doses (1, 10 and 20 ppm) of the insecticide, the population was reduced by 46.6 to 92.8% of control after 1 day while the population was reduced by 18.2 to 58.5% after 5 days treatment. L a l et al. (1987 b) noticed similar recovery of *Tetrahymena* from the toxic effect of DDT, fenitrothion and chlorpyrifos with time. This phenomenon is described as recovery (F r a n k e l 1965), or adaptation (R o b e r t s and O r i a s 1974). It is assumed that recovery of the organisms is obtained through increased resistance to chemicals. This could be achieved through two physiological reactions in the cells: (a) by lowering the inhibitory action of chemicals through active depletion and/or (b) through increased activity of the physiological systems affected by the chemicals (K i e r s n o w s k a 1982).

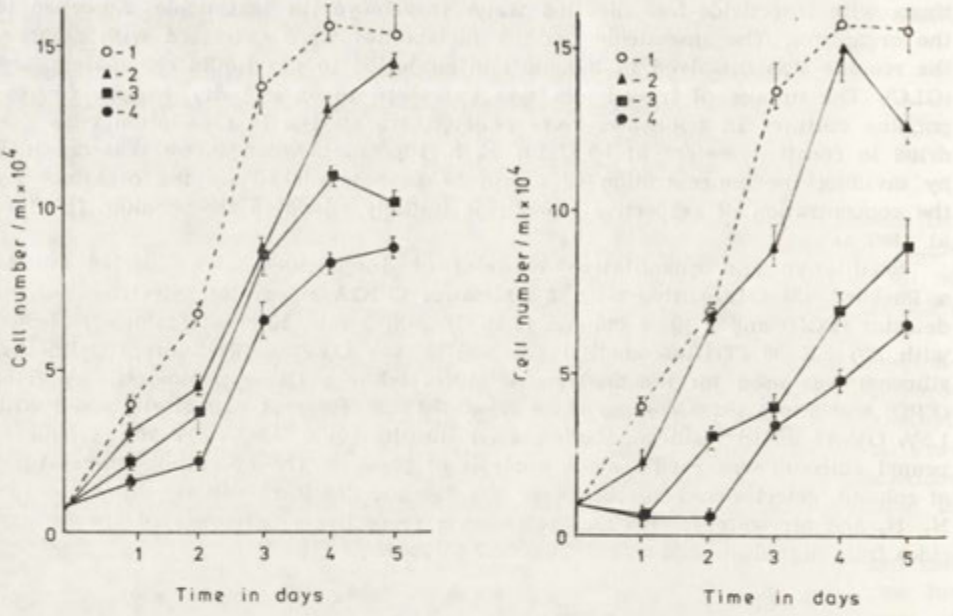

Fig. 1. Effect of aldrin on the growth of *Tetrahymena pyriformis*, 1 — control, 2 — 10 ppm, 3 — 20 ppm, 4 — 50 ppm

Fig. 2. Effect of phorate on the growth of *Tetrahymena pyriformis*, 1 — control, 2 — 1 ppm, 3 — 10 ppm, 4 — 20 ppm

In cultures treated with higher doses of aldrin (100 ppm) and phorate (50 ppm) the organisms lost their pear-shape, became round and immobile. Light microscopic observations revealed extrusion of mucocysts. Further, cell lysis took place within 6 h of incubation. Such changes in the morphology of *Tetrahymena* under the influence of toxic chemicals have been reported by Rohatagi and Krawiec (1973) and Gray and Kennedy (1974). Mucocyst discharge by *Tetrahymena* appears to be a non-specific protective mechanism (Schultz and Dumont 1977) and mucous discharge could serve to protect the cell surface of the organisms as well as to bind toxic chemicals.

Uptake of aldrin by *T. pyriformis* is shown in Table 2. As the organisms metabolized aldrin to dieldrin the uptake was expressed as sum total of aldrin and dieldrin. Uptake of total aldrin increased with the increase in insecticide concentration in the culture medium and ranged from 32.1 to 924.3 ppm at 0.1, 0.5 and 1 ppm doses (Table 2). *T. pyriformis* was less efficient in picking up phorate and its concentration ranged from 21.1 to 948.1 ppm from the culture medium containing 2.5, 5 and 10 ppm of the insecticide (Table 3). Bioconcentration factor for aldrin was several fold higher than that of phorate in *T. pyriformis*.

Table 2

Uptake (μg g^{-1} dry weight) of aldrin (aldrin + dieldrin) by *Tetrahymena pyriformis* (± SE)

Exposure (h)	Intitial aldrin concentration (μg ml^{-1}) in medium		
	0.1	0.5	1.0
2	32.1±0.9 (321)*	137.9±3.4 (276)	343.5±6.6 (344)
4	52.0±2.8 (520)	147.1±6.0 (294)	447.9±22.8 (448)
6	89.5±4.2 (895)	407.2±4.9 (814)	757.9±14.1 (758)
8	117.1±3.7 (1171)	272.9±0.5 (546)	924.3±40.8 (924)
10	56.1±5.9 (561)	146.8±1.7 (294)	487.9±45.7 (488)
12	97.2±0.3 (972)	179.8±9.5 (360)	536.2±33.8 (536)

* Values in parantheses indicate bioconcentration factor.

Table 3

Uptake (μg g^{-1} dry weight) of phorate by *Tetrahymena pyriformis* (± SE)

Exposure (h)	Initial phorate concentration (μg ml^{-1}) in the medium		
	2.5	5	10
2	21.1±1.2 (8.4)*	107.9±2.0 (22)	224.1±22.1 (22)
4	37.7±5.7 (38)	52.6±0.4 (11)	361.7±60.8 (36)
6	36.4±3.7 (15)	117.5±17.0 (36)	948.1±48.2 (95)
8	85.1±9.8 (34)	184.7±8.4 (37)	671.9±61.8 (67)
10	30.6±4.1 (12)	135.4±15.9 (27)	544.3±37.5 (54)
12	72.6±1.4 (29)	231.3±2.1 (46)	511.9±52.9 (51)

* Values in parantheses indicate bioconcentration factor.

Aldrin was bioconcentrated 1171, 814 and 924 folds from the media containing 0.1, 0.5 and 1.0 ppm of the insecticide (Table 2). On the other hand, phorate was bioconcentrated 38, 37 and 95 fold from the media containing 2.5, 5 and 10 ppm respectively of the insecticide (Table 3). Uptake of insecticides by microorganisms has been inversely related to their water solubility (L a l 1984, L a l et al. 1987 a). The water solu-

bility of aldrin is 0.027 ppm at 25°C whilst that of phorate is 50 ppm at 20°C. A low water-solubility and the high lipophilic nature of aldrin may have enhanced its potential to accumulate in *T. pyriformis* in comparison to phorate.

Patterns of aldrin uptake showed that the maximum concentration of the insecticide in *T. pyriformis* reached after 6 to 8 h of exposure (Table 2). A similar pattern was also noticed with phorate at 2.5 and 10 ppm whilst phorate uptake continued to increase till 12 h at 5 ppm treatment (Table 3). However, a much more rapid uptake of insecticides has been reported in other organisms. *Agrobacterium tumefaciens* required only 15 min to uptake 90-100% of DDT and dieldrin from the medium (Chacko and Lockwood 1967) whilst streptomycetes and a fungus, *Trichoderma*, respectively accumulated 80% and 76% of dieldrin in the same time.

T. pyriformis was not very efficient in metabolizing insecticides. Though, aldrin was converted to dieldrin, only a trace amount of this metabolite was detected (Table 4). Phorate was unaffected by the organism and chromatograms of treated samples revealed only one peak pertaining to the parent compound. There are reports that fenitrothion, chlorpyrifos, malathion and endosulfan were accumulated but not metabolized by *T. pyriformis* (Lal et al. 1987 b; Tandon et al. 1988). Therefore, bioconcentration of these insecticides in higher amounts by protozoans may prevent their subsequent degradation by other biotic and abiotic means and enhance their biomagnification in higher organisms.

Table 4

Dieldrin concentration (μg g^{-1} dry weight) in *Tetrahymena pyriformis* exposed to 0.1, 0.5 and 1 ppm of aldrin for 12 h (±SE)

Exposure (h)	Initial aldrin concentration in the culture medium (μg ml^{-1})		
	0.1	0.5	1.0
2	0.13±0.01	0.82±0.23	1.67±0.18
4	0.94±0.12	1.31±0.05	1.98±0.05
6	0.84±0.05	1.60±0.08	3.23±0.56
8	0.65±0.04	2.59±0.09	3.66±0.38
10	0.71±0.08	0.67±0.07	2.36±0.59
12	0.95±0.15	1.01±0.03	1.88±0.06

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REFERENCES

Butler G. L. 1977: Algae and pesticides. Res. Rev., 66, 19-62.
Chacko C. L. and Lockwood J. L. 1967: Accumulation of DDT and dieldrin by microorganisms. Can. J. Microbiol., 11, 1123-1126.
Frankel J. 1965: The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. J. Exp. Zool., 159, 113-148.
Gray J. P. and Kennedy J. R. 1974: Ultra and physiological effects of non-tobacco cigarettes on *Tetrahymena*. Arch. Environ. Health, 28, 283-291.
Johannes R. E. 1968: Advances in Microbiology in Sea. Academic Press, New York, p. 203.
Kiersnowska M. 1982: Endogenous recovery of cell divisions in the presence of cycloheximide by the ciliate *Chilodonella cucullulus*. Experientia, 38, 924-927.
Lal S. 1984: Insecticide Microbiology. Springer-Verlag. Berlin, p. 61.
Lal R. and Saxena D. M. 1982: Accumulation, metabolism and effects of organochlorine insecticides on microorganisms. Microbiol. Rev., 46, 95-127.
Lal S., Lal R. and Saxena D. M. 1987 a: Bioconcentration and metabolism of DDT, fenitrothion and chlorpyrifos by the blue-green algae *Anabaena* sp. and *Aulosira fertilissima*. Environ. Pollut., B. 46, 187-196.
Lal S., Saxena D. M. and Lal R. 1987 b: Uptake, metabolism and effects of DDT, fenitrothion and chlorpyrifos on *Tetrahymena pyriformis*. Pestic. Sci. 21, 181-191.
Roberts Ch. T. and Orias E. 1974: On the mechanism of adaptation to protein synthesis inhibitors by *Tetrahymena*. Facilitation, cross adaptation and resensitization. J. Cell Biol., 62, 707-716.
Rohatagi K. and Krawiec S. 1973: Some effects of chloramphenicol and ethidium bromide on *Tetrahymena pyriformis*. J. Protozool., 20, 425-430.
Schultz T. W. and Dumont J. N. 1977: Cytotoxicity of synthetic fuel products on *Tetrahymena pyriformis*. I. Phenol. J. Protozool., 24, 164-172.
Tandon R. S., Lal R. and Narayana Rao V. V. S. 1988: Uptake and effects of malathion and endosulfan on *Tetrahymena pyriformis*. Pesticides (India) (in press).
Williams P. P. 1977: Anaerobic metabolism of pesticides. Res. Rev., 66, 63-135.

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The Relationship Between the Respiratory Rate and the Period of the Contraction-Relaxation Cycle in Plasmodia of *Physarum polycephalum*

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Synopsis. Starvation of *Physarum* plasmodia provokes their transition into the dormant structures. This is accompanied by a decline of the respiratory rate and a prolongation of the period of the contraction-relaxation cycle of plasmodial actomyosin. The respiration decreases in jump-like manner while the prolongation of the period shows the sigmoid dependence upon the time of starvation. Plasmodial mitochondria exhibit two pathways of the electron transport: (1) the cytochrome pathway which is cyanide-sensitive, and (2) the alternative one which is inhibited by SHAM (salicylhydroxamic acid). During starvation the cyanide-resistant respiration declines in non-monotonous manner with the local maximum around 4-6 h while the period of the contraction-relaxation cycle increases monotonously in the presence of KCN. In starvation medium without the respiratory inhibitors and in the presence of SHAM the kinetics of the respiratory rate decline are very similar. However, in contrast to cyanide, SHAM evokes large variations of the oscillations period in course of starvation. The values of the respiration rate and the contraction-relaxation period which were measured at the same time of starvation form the ordered pairs. These pairs determine the relationship between both plasmodial activities. In such defined plane, the obtained data form two disjoint sets with the demarcation respiratory rate of about 15 $nAtO_2min^{-1}mg^{-1}$. Above the demarcation value, the respiratory rate and the oscillations period show Z-shape relationship, i.e., they are not in one-to-one correspondence. At the lower respiratory rate, i.e., below 15 $nAtO_2min^{-1}mg^{-1}$, the linear relationship exists. In this case, the decrease of the respiration is accompanied by the prolongation of the period. The pre-

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sented relationships were obtained in the indirect way and the advantages and the shortcomings of this method are described. The different forms of the relationship between the respiration and the period of the contraction-relaxation cycle are discussed in respect to the mechanism of oxygen reduction and the energetic state of plasmodia.

Uncoupling of the nuclear division from the cytokinesis leads to the formation of a single cell containing a lot of nuclei. Such a cellular organization is known as plasmodium and exists in the life cycle of a true slime mold *Physarum polycephalum*. This cellular organization of *Physarum* is often referred to as a syncytial form because plasmodia show the strong tendency to fuse with one another (Sauer 1986). The size of plasmodia may differ in wide range from 0.1 mm in diameter in submerged shaken culture (microplasmodia) up to 0.1 m^2 or more, in case of macroplasmodia in the surface culture. The large size of the plasmodia, as compared to the other non-muscle cells, enables to study their contractile properties with the aid of tensiometric methods (Kamiya 1970). *Physarum* actomyosin contracts and relaxes spontaneously with a period which varies within the range of a few minutes showing a complex oscillatory pattern (Kamiya and Yoshimoto 1972). It is evident that the complex pattern of the contraction-relaxation cycle revealed by tensiometry reflexes the dynamics of the intracellular metabolic processes in plasmodium. The problem is how to draw out these informations from the tensiograms. The present knowledge allows to do it in a very limited range (Wohlfarth-Bottermann 1986).

The purpose of the present investigations was to check whether the respiratory activity of plasmodia and the period of their contraction-relaxation cycle are related to each other. Some earlier results have suggested the existence of a relation between both plasmodial activities. The disturbances in the mitochondrial functions lead to changes in the pattern of the oscillations of the contractile force (Satoh et al. 1982, 1984, Korohoda et al. 1983, Shraideh et al. 1983, Baranowski et al. 1983), or even to the reversible cessation of the oscillatory rhythm (Baranowski 1985). The latter effect is observed in response to the blockage of two pathways of electron transport in plasmodial mitochondria. Like in higher plants and other microorganisms, the plasmodial mitochondria exibit both the cytochrome pathway and the alternative one which branches off at the level of ubiquinone (Henry and Nyns 1975, Akimenko 1981, Laties 1982, Rychter 1982).

Simultaneous registration of the respiratory and contractile activity of the same plasmodium is very difficult in practice. To overcome this problem we have decided to use an indirect method. Both activities were registered as a function of time of starvation of the same population of

the microplasmodia. The advantage of the method is that during starvation the microplasmodia reduce their respiration without treatment with respiratory inhibitors (Allen et al. 1985). The shortcoming of the method is that the registration of the contraction-relaxation cycle must be carried out on macroplasmodia with a time delay indispensable for fusion of microplasmodia which are too small for tensiometry. From physiological point there is no difference between micro- and macroplasmodia. Starvation stimulates the encystment of both plasmodial forms. However, they react to starvation with a different rate, probably due to the divergence of the surface/volume ratio. In the starvation medium the microplasmodia form dormant structures (spherules) after about 18-30 h, while starved macroplasmodia migrate several days before they form a large cluster of spherules — sclerotium. (Raub and Aldrich 1982). Thus, the differentiation of macroplasmodia is strongly retarded comparing to the same process in microplasmodia. However, it is difficult to judge whether in the period of the fusion (1-3 h) the rate of the differentiation of microplasmodia is as fast as in shaken, starvation medium. We have assumed that during fusion of microplasmodia the stage of their differentiation does not change significantly. Therefore the results of tensiometric measurements are presented against the age of microplasmodia which were taken for the formation of syncytium. Hereafter, the duration of starvation is often referred to as the age of micro- or macroplasmodia.

The presented results show that the frequency of the contraction-relaxation cycle is a function of the rate of oxygen consumption only at the low level of the respiration. This conclusion is valid even if we assume that the differentiation proceeds in the period of fusion of microplasmodia.

Material and Methods

Microplasmodia of *Physarum polycephalum*, strain USSR MCC were grown on the semi-defined medium according to Daniel and Baldwin (1964). At the cell density of 0.1 ml of plasmodial pellet per 1 ml of culture medium i.e., after about 48 h of growth, the microplasmodia were sedimented at the low speed (300 g) for 1 min and washed twice with the cold starvation medium. As the starvation medium (SM) there was used 3× diluted standard salt solution (Daniel and Baldwin 1964). The pellet was resuspended with 20 vol of SM and allowed to starve with permanent agitation. Every 2 h an aliquot of microplasmodial suspension was isolated for the respiration and tensiometric assays. For this purpose the microplasmodia were washed and diluted with 2 vol of salt solution (SS) containing 1 mM $CaCl_2$, 3 mM $MgCl_2$, 2 mM NaCl and 1 mM KCl, pH 7.0. The rate of oxygen uptake was measured at the cell density of

0.04 ml of plasmodial pellet per 1 ml of SS with the aid of Clark type electrode. The aliquot taken for the tensiometry (150 µl of plasmodial suspension) was placed on a filter paper in the area limited by a plastic ring (0.8 cm in diameter) and kept in a moist chamber as to get plasmodial samples (Kincaid and Mansur 1978). The tensiometric measurements started 1-3 h later i.e., just after fusion of the microplasmodia and the formation of macroplasmodium. The contraction-relaxation cycle was registered by means of the contact method (Wohlfarth-Bottermann 1975) in a chamber equipped with Peltier element and a set for the replacement of test solutions. The test solutions containing KCN or SHAM (salicylhydroxamic acid) dissolved in SS were adjusted to pH 7.0. All procedures were carried out at 20°C with protection of the microplasmodia from the light.

Chemicals: pepton, yeast extract, glucose — Difco Laboratories, Detroit, Michigan; hemin, salicylhydroxamic acid (SHAM) — Sigma Chemical Co., St. Louis, Missouri; salts analytical grade.

Results

The strain of *Physarum polycephalum* used in the presented investigations shows satisfactory synchronous conversion of microplasmodia into spherules. Following this process by means of light microscopy the first symptoms of the spherule formation are observed after 14 h of starvation. The process is completed after 18 h and its kinetics was shown elsewhere (Beylina et al. 1988).

As it was outlined in Introduction the fusion of microplasmodia strongly retards the differentiation of *Physarum*. This effect is clearly seen in relatively "young" microplasmodia. Up to 4 h of starvation the fusion of microplasmodia is completed after 1 h and the macroplasmodium migrates about 3 days before sclerotium is formed. Within the range of 6-8 h of starvation these processes last 1.5-2 h and 2 days respectively. The microplasmodia starved for 10-12 h form syncytium after 3 h. In this case the macroplasmodium usually does not migrate and spherulation is completed after 5-8 h. The microplasmodia starved longer than 12 h do not fuse during at least 2 days. The macroplasmodium develops after that period. However, in all probability, previously formed spherules are its origin.

During starvation the microplasmodia reduce their rate of oxygen consumption (see Fig. 1). This phenomenon was already described but a monotonous decline of the respiration was observed (Allen et al. 1985). Figure 1 shows that during the first 4 h after the transfer of microplasmodia to starvation medium their respiration rate is almost constant, sharply declines between 4 h and 6 h and finally achieves a new, steady level. It suggests the existence of two dynamically stable states of the respiratory activity. The starvation switches-over the activity to

the level about 3 fold lower (see Fig. 1). In other words, in early phase of starvation the respiration of plasmodia is controlled by a system which shows trigger properties.

The period of the contraction-relaxation cycle increases during starvation. Figure 1 presents the sigmoid dependence of the period on time of transfer of microplasmodia to the starvation medium. The age of plasmodia was suspected to affect the pattern of the contraction-relaxation cycle but there was lack of clear evidence of this fact. It was only shown that starvation prolongates the re-uptake of the oscillations in *Physarum* endoplasmic drops and modifies the kinetics of this transition (B a r a n o w s k i 1980).

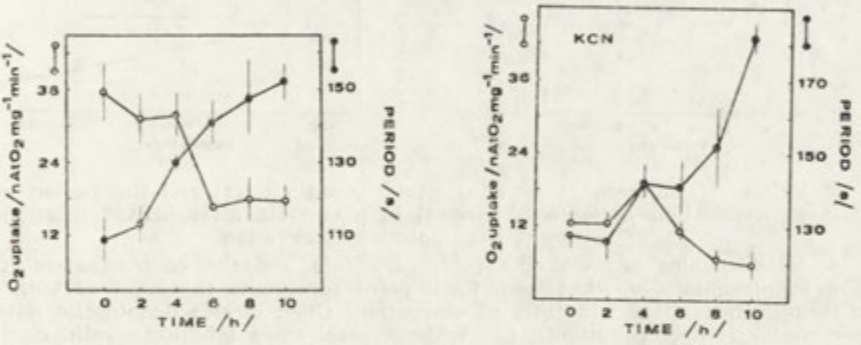

Fig. 1. Changes in the rate of oxygen uptake (open circles) and in the period of the contraction-relaxation cycle (solid circles) in course of starvation of *Physarum polycephalum* plasmodia. Starvation time — in hours

Fig. 2. Values of the respiratory rate (open circles) and the period of the contraction-relaxation cycle (solid circles) in plasmodia after KCN treatment at different time points of starvation

The respiration of *Physarum plasmodia* is partially cyanide resistant. The alternative pathway of the respiration in *Physarum* is effectively inhibited by salicylhydroxamic acid — SHAM (B e y l i n a et al. 1988). The values of both contractile and respiratory activities, in plasmodia of different age in the presence of cyanide, are shown in Fig. 2. In contrast to the observations of A l l e n et al. (1985) we have registered a decrease of cyanide-resistant respiration in non-monotonous manner with maximum around 4-6 h of starvation. These changes of the respiration are accompanied by prolongation of the period of oscillations (see Fig. 2). Comparing of Fig. 1 and Fig. 2 it is easy to notice that cyanide decreases the frequency of the oscillations. This effect was already observed by K o r o h o d a et al. (1983).

In the early phase of starvation the treatment of plasmodia with SHAM evokes changes in the period of the contraction-relaxation cycle after about 20 min. For this reason the measurement of respiration

has started since the 20th min of SHAM treatment (Fig. 3). The SHAM-resistant respiration (Fig. 3) and the control respiration (Fig. 1) change similarly during starvation. However, in contrast to the changes of the period under control conditions (Fig. 1) or in the presence of cyanide (Fig. 2), the treatment of plasmodia with SHAM evokes large variations of the frequency in course of starvation.

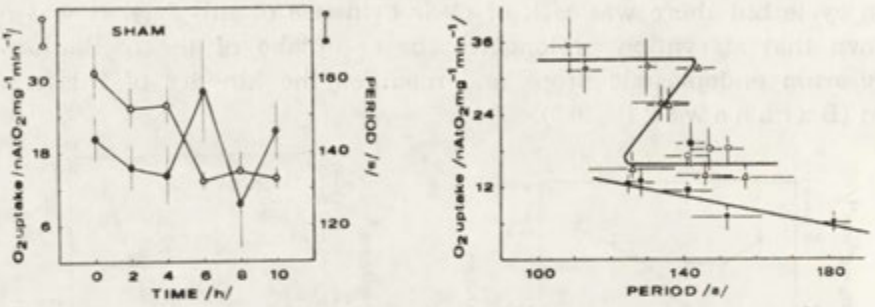

Fig. 3. Values of the rate of oxygen uptake (open circles) and the period of the contraction-relaxation cycle (solid circles) in plasmodia after SHAM treatment at different time points of starvation

Fig. 4. Relationships between the repiratory rate and the contraction-relaxation period in plasmodia of *Physarum*. Each point represents the level of both plasmodial activities at certain time of starvation. Open circles denote the data obtained under control conditions, i.e., without respiratory inhibitors, solid circles — under 5 mM KCN, triangles — under 7 mM SHAM treatment

The data presented in Fig. 1, 2 and 3 enable to work out a relationship between the respiration of plasmodia and the period of oscillations of the force which is generated by plasmodial actomyosin. These data are summarized in Fig. 4. Each point in Fig. 4 represents both plasmodial activities which were registered at the same time of starvation. The collection of the data in Fig. 4 may be arranged in two sets. The first one is mainly represented by the data obtained under control conditions (Fig. 1) and in the presence of SHAM (Fig. 3). The elements of this set determine Z-shape of the relationship. It denotes that in the region of hysteresis, the same frequency of the contraction-relaxation cycle may correspond to different rates of oxygen uptake. Outside this region, the high level of the respiratory activity corresponds to high frequencies of the oscillations; the low level of the respiration corresponds to low frequencies. However, within the range of both high and low frequencies the variations of the period may occur without detectable changes in the rate of the respiration.

The second set of the data in Fig. 4 is represented by the results of measurements carried out in the presence of cyanide (Fig. 2). The experimental points are satisfactory approximated by a straight line. It

means that the period of oscillations and the respiration rate are in one-to-one correspondence. The decrease of the respiratory activity is accompanied by the prolongation of the period of the oscillations.

Discussion

The presented investigations have revealed two different relationships between the contractile and the respiratory activity (see Fig. 4). The Z-shape correlation is observed for the respiratory rates higher than 15 $nAtO_2mg^{-1}min^{-1}$ while below this value the linear relationship exists. On the other hand, the linear correlation is observed when only the alternative pathway of respiration operates while under control conditions and in the presence of SHAM the correlation shows the Z-shape form. It suggests that the appearance of these two different forms of the correlation should be considered with respect to: 1) the mechanism of oxygen reduction, and 2) the energetic state of plasmodia.

The processes which lead to the reduction of oxygen via the cytochrome chain or the alternative pathway are probably essentially different. Rustin et al. (1984) have proposed that free radicals reactions involving superoxide and membrane lipid radicals are engaged in the transport of the electrons via the alternative pathway. This hypothesis implies the changes in the antioxidant defense processes in response to activation of the cyanide-resistant respiration. These processes depend upon several isozymes of superoxide dismutase (SOD), catalase and glutathione peroxidase. Indeed, the increase in the mangano-isozyme of SOD and the decrease in glutathione (GSH) concentration was observed during starvation of plasmodia (Allen et al. 1985, Nations et al. 1987). However, considering the mechanism of oxygen reduction we are not in position to give a satisfactory explanation of the different forms of the relationships between the contractile and respiratory activity because the point which represents both plasmodial activities, at the time of the highest level of the cyanide-resistant respiration (Fig. 2), does not belong to the linear relationship, but lies at the lower branch of Z-shape characteristic (see Fig. 4).

Similarly, the considerations of the energetic aspect of the presented results do not lead to the univocal conclusion. It may be assumed that the different shapes of the relationship shown in Fig. 4 depend upon the initial level of oxygen uptake with the demarcation respiratory rate of about 15 $nAtO_2mg^{-1}min^{-1}$ which delimits two different energetic states of plasmodia. Reich and Sel'kov (1981) have considered minimal metabolism of the cell evoked by a nutrient shortage. Under this condition, the cell preserves only "vital reactions" i.e., the processes

which are indispensable for the cell to survive the starvation. In these circumstances, all ATPase-like reactions which are regulated by their products via the negative feed-back mechanism have to be reduced. In the early phase of starvation the microplasmodia reduce their glycogen content (Raub and Aldrich 1982). Schreckenbach et al. (1981) have shown that during starvation, glucan as a structural component of the spherules, and the disacharide trehalose as their energy reservoir are the main products of glucose breakdown. Studies of the intact plasmodia by means of ^{31}P-nuclear magnetic resonance did not show the presence of phosphocreatinine and phosphoarginine (Kohama et al. 1984). Therefore, it seems to be likely that the oxidative phosphorylation determines the energetic state of plasmodia during starvation. The drop in the rate of respiration observed after 4-6 h of starvation (Fig. 1) denotes the decrease of ATP production. This decline may be even sharper if we assume that the alternative pathway is active under control conditions. Our previous investigations did not solve the last problem definit (Beylina et al. 1988). Figure 4 shows that this decline of the energy state is not sufficient to change the form of the correlation between the respiratory and the contractile activity in plasmodia. At the beginning of these considerations we have outlined that during starvation glycolysis does not participate in ATP production. Under this condition, the additional blockage of the cytochrome oxidase suggests that the alternative pathway is a sole source of ATP. However, the alternative pathway is not able to maintain the integrity of plasmodia provide it is not supported by glycolysis or extracellular respiratory substrates (Korohoda et al. 1983, Baranowski 1985). It leads to the conclusion that the treatment of plasmodia with KCN stimulates glycolysis. Hence, it is doubtfull whether the linear correlation between the respiration and the period of the contraction-relaxation cycle is unmasked due to an increase of the energetic stress.

It is most probable that in the intact plasmodia the period of the contraction-relaxation cycle is not controlled by a single factor. Theoretical considerations strongly suggest that the period, the amplitude and the level of the force oscillations are dependent upon the steady-states between the different agreggation forms and the arrangement patterns of plasmodial actin (data unpublished). These steady-states, in turn, are controlled by the complex system of the regulatory proteins (Hinssen et al. 1986, Hatano 1986, Hatano et al. 1987). Our results suggest that the system of the period regulation is simplified at the low rates of respiration. Under these conditions, the period of the contraction-relaxation cycle can be interpreted in terms of the respiratory rate.

REFERENCES

Akimenko V. K. 1981: Cyanide-resistant respiration in microorganisms. Usp. Mikrobiol. 16, 3-30.

Allen R. G., Newton R. K., Sohal R. S., Shipley G. L. and Nations C. 1985: Alterations in superoxide dismutase, glutathione, and peroxides in the plasmodial slime mold *Physarum polycephalum* during differentiation. J. Cell. Physiol., 125, 413-419.

Baranowski Z. 1980: Kinetics of the regeneration of rhythmic contraction activity in *Physarum polycephalum* drops. Acta Protozool., 19, 67-76.

Baranowski Z. 1985: Consequences of impeding in mitochondrial functions in *Physarum polycephalum*. III. Reversible cessation of the contraction-relaxation cycle and the temperature sensitivity of the alternate respiratory pathway. Europ. J. Cell Biol., 39, 283-289.

Baranowski Z., Shraideh Z., Korohoda W. and Wohlfarth-Bottermann K. E. 1983: Consequences of impeding in mitochondrial functions in *Physarum polycephalum*. II Influence on "de novo" generation of contractile activities and responses to glucose and blue light. Europ. J. Cell Biol., 31, 187-196.

Beylina S. I., Hrebenda B., Cieślawska M. and Baranowski Z. 1988: Cyanide-resistant respiration of *Physarum polycephalum* in course of starvation. Cell Biol. Int. Reps., 12, 29-34.

Daniel J. W. and Baldwin H. H. 1964: Methods of culture for plasmodial *Myxomycetes*. Methods in Cell Physiol., 1, 9-41.

Hatano S. 1986: Actin, myosin, and the associated-proteins from the Physarum plasmodium. In: The Molecular Biology of *Physarum polycephalum* (eds. W. F. Dove, J. Dee, S. Hatano, F. B. Haugli and K. E. Wohlfarth-Bottermann), Plenum Press and NATO Scientific Affairs Division, New York and London, 165-174.

Hatano S., Ishigami M., Uyeda T. Q. P., Sonobe S. and Kuroda K. 1987: Regulation of actin polymerization in *Physarum polycephalum* and *Amoeba proteus*. In: Nature and Function of Cytoskeletal Proteins in Motility and Transport, Fortschritte der Zoologie, 34, (ed. Wohlfarth-Bottermann), Gustav Fischer Verlag, Stuttgart, N. York, 41-52.

Henry M. F. and Nyns E. J. 1975: Cyanide-insensitive respiration. An alternative mitochondrial pathway. Subcell. Biochem., 4, 1-65.

Hinssen H., Engels F. E. and D'Haese J. 1986: Kinetics of modulator-actin interactions: a comparison of *Physarum* fragmin with action modulators from different muscle types. In: The Molecular Biology of *Physarum polycephalum* (eds. W. F. Dove, J. Dee, S. Hatano, F. B. Haugli and K. E. Wohlfarth-Bottermann), Plenum Press and NATO Scientific Affairs Division, New York and London, 199-206.

Kamiya N. 1970: Contractile properties of the plasmodial strand. Proc. Japan Acad., 46, 1026-1031.

Kamiya N. and Yoshimoto 1972: Dynamic characteristics of the cytoplasm. A study on the plasmodial strand of a myxomycete. In: Aspects of Cellular and Molecular Physiology. (Ed. K. Hamaguchi), Univ. Tokyo Press, Tokyo, 167-189.

Kincaid R. L. and Mansour T. E. 1978: Measurement of chemotaxis in the slime mold *Physarum polycephalum*. Exp. Cell Res., 116, 365-375.

Kohama K., Tanokura M. and Yamada K. 1984: ^{31}P-nuclear magnetic resonance studies of intact plasmodia of *Physarum polycephalum*. FEBS Letters, 176, 161-165.

Korohoda W., Shraideh Z., Baranowski Z. and Wohlfarth-Bottermann K. E. 1983: Energy metabolic regulation of oscillatory contraction activity in *Physarum polycephalum*. Cell Tiss. Res., 231, 675-691.

Laties G. G. 1982: The cyanide-resistant, alternative path in higher plant respiration. Ann. Rev. Plant Physiol., 33, 519-555.

Nations C., Allen R. G., Balin A. K., Reimer R. J. and Sohal R. S. 1987: Superoxide dismutase activity and glutathione concentration during the calcium-induced differentiation of *Physarum polycephalum* microplasmodia. J. Cell. Physiol., 133, 181-186.

Raub T. J. and Aldrich H. C. 1982: Sporangia, spherules, and microcysts. In: Cell Biology of *Physarum* and *Didymium*. (Eds. H. C. Aldrich and J. W. Daniel), vol. II, Academic Press, New York, London, 21-75.

Reich L. G. and Sel'kov E. E. 1981: Energy Metabolism of the Cell. Academic Press, London, New York, Toronto, Sydney, San Francisco, 1-345.

Rustin P., Dupont J. and Lance C. 1984: Involvement of lipid peroxy radicals in the cyanide-resistant electron transport pathway. Physiol. Veg., 22, 643-663.

Rychter A. M. 1982: Alternative respiratory pathway in higher plants. Post. Biochem., 28, 89-111.

Satoh H., Ueda T. and Kobatake Y. 1982: Primary oscillator of contraction rhythm in the plasmodium of *Physarum polycephalum*: Role of mitochondria. Cell Struct. Funct., 7, 275-283.

Satoh H., Ueda T. and Kobatake Y. 1984: Rhythmic contraction in the plasmodium of the myxomycete *Physarum polycephalum* in relation to the mitochondrial function. Cell Struct. Funct., 9, 37-44.

Sauer H. W. 1986: Introduction to *Physarum*. In: The Molecular Biology of *Physarum polycephalum* (eds. W. F. Dove, J. Dee, S. Hatano, F. B. Haugli and K. E. Wohlfarth-Bottermann), Plenum Press and NATO Scientific Affairs Division, New York and London, 1-17.

Schreckenbach T., Walckhoff B. and Verfuerth C. 1981: Blue-light receptor in a white mutant of *Physarum polycephalum* mediates inhibition of spherulation and regulation of glucose metabolism. Proc. Natl Acad. Sci. USA, 78, 1009-1013.

Shraideh Z., Baranowski Z., Korohoda W. and Wohlfarth-Bottermann K. E. 1983: Consequences of impeding in mitochondrial function in *Physarum polycephalum*. I. Reversible effects of anoxia, KCN, and influence of the Ca^{2+} ionophore A-23187. Eur. J. Cell Biol., 31, 175-186.

Wohlfarth-Bottermann K. E. 1975: Tensiometric demonstration of endogenous oscillating contractions in plasmodia of *Physarum polycephalum*. Z. Pflanzenphysiol., 76, 14-27.

Wohlfarth-Bottermann K. E. 1986: Biological aspects of motility. In: The Molecular Biology of *Physarum polycephalum*, (eds. W. F. Dove, J. Dee, S. Hatano, F. B. Haugli and K. E. Wohlfarth-Bottermann), Plenum Press and NATO Scientific Affairs Division, New York and London, 165-174.

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Impact of the Simultaneous and Successive Imprinting of Different Peptide Molecules on Receptor "Memory" in *Tetrahymena*

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Synopsis. The imprinting induced in *Tetrahymena* with di-, oligo- or polypeptides did not appreciably decrease when these molecules were applied in combination either simultaneously, or in succession at 24-h intervals. It follows that the unicellular *Tetrahymena* is capable of developing a receptor-level "memory" simultaneously for several peptides, i.e., exposure to a "new" peptide does not extinguish the "memory" of a previous interaction with another peptide, if the time interval between the interactions is long enough. In the given conditions of experiment, simultaneous treatment with several peptides had a distinct stimulant effect on imprinting. The influence of peptide treatments lasted long, and in part of the cases it was even more pronounced one to four weeks after treatment than one day after it. Some peptides, which produced either no imprinting or a negative imprinting after one day, did develop a positive imprinting effect later on.

Hormonal imprinting occurs at the primary interaction of a given hormone with the target cell, and results in a lifelong alteration of cellular response to, and binding capacity for, the hormone, which is transmitted to many progeny cell generations (Csaba 1980, 1981). In higher organisms, hormonal imprinting occurs in the perinatal period (Csaba 1986 a), and unicellular organisms, too, show the phenomenon of imprinting in experimental conditions (Csaba 1985, 1986 b) on exposure to a foreign molecule which develops action at receptor level, and gives thereby rise to formation of a receptor from non-specific membrane patterns. The imprinted cell "remembers" the primary interaction for lifetime, and even transmits the "memory" of hormonal imprinting to as many as 500 progeny generations (Csaba 1982). The receptor "memory" resembles neuronal memory inasmuch as it is either dimin-

ished or extinguished, if another strong impulse follows immediately upon the primary one, but becomes amplified if the second impulse follows later (Csaba et al. 1984 a).

In the present study we made attempt at assessing the scope of imprinting-associated memory in unicellulars, more precisely the impact of multiple, simultaneous or successive imprinting relative to that effected by a single peptide. We used for imprinting peptide molecules of different sizes (dipeptide, oligopeptide, polypeptide and glycoprotein), to obtain information also on possible interrelationships of the effect of simultaneous or successive imprinting with the molecular size.

Material and Methods

Tetrahymena pyriformis GL cells, maintained in 1 per cent Bacto tryptone medium (Difco, Michigan, U.S.A.) containing 0.1 per cent yeast extract at 28°C were treated in the logarithmic phase of growth with the following peptides:

(1) insulin (Semilente, MC, Novo, Copenhagen);
(2) thyrotropin (TSH, Ambinon, Organon, Oss);
(3) oxytocin (Richter, Budapest);
(4) leucine-tryptophane-methionine-arginine-phenylalanine formiate oligopeptide (Serva, Heidelberg);
(5) alanine-tyrosine dipeptide (Serva, Heidelberg).

All peptides were applied in 10^{-6}M concentration.

Simultaneous Treatments

One mass culture which was not treated on any occasion served as control. One mass culture each was treated with peptide No. 1, 2, 4 or 5 alone, and a fifth mass culture was treated with all four peptides simultaneously. The treatment always lasted 4 h, after which the cells were returned to plain medium for 24 h, and fixed. Other mass cultures were maintained under oil overlay as follows: A 1 cm long piece of the small intestine of a starved rat and 0.5 ml paraffine oil were added to 10 ml distilled water (Williams et al. 1980 a), and this system was sterilized under 1 at pressure at 121°C. The *Tetrahymena* cells were inoculated under the oil film formed on the surface of the nutrient fluid after cooling, were cultured in the dark at 28°C for 1, 2 or 4 weeks, were returned to Bacto tryptone medium for 2 days, and fixed.

Successive Treatments

Apart from an untreated mass culture serving as absolute control, one culture each was treated on a single occasion with peptide No. 1, 2, 3 or 5 for use as peptide control. The experimental cultures were treated with peptides No. 1, 2, 3 and 5 on four consecutive days, in the following two sequences:

(1) dipeptide, oxytocin, insulin, TSH;
(2) TSH, insulin, oxytocin, dipeptide.

In this series, too, the cells were returned to plain medium for 24 h and fixed. Other mass cultures had been maintained and fixed as above.

Hormone Binding Studies

After fixation (4 per cent formaline solution in PBS, for 5 min) the cells were washed in several changes of PBS, and were incubated in presence of fluoresceine isothiocyanate- (FITC, BDH, England) — labeled peptide (insulin, TSH, oxytocin or oligo- or dipeptide) for 1 h at room temperature. The FITC/protein ratios of the conjugates were the following: insulin 0.42; TSH 1.83; oxytocin 1.47; oligopeptide 0.32; dipeptide 0.29. The protein concentration of the conjugate was always 0.05 mg/ml.

After incubation the cells were washed in three changes of PBS, spread on slides, dried and examined for intensity of fluorescence with a Zeiss Fluoval cytofluorimeter, which was connected with a digital processor for signal transformation. The digital signals were recorded by a Hewlett Packard HP41CX calculator and were analysed for mean values, standard deviation and significance of inter-group variation (by Student's t-test and analysis of variance). Each experiment was performed in three replicates, and 20 cells were assayed for fluorescence in each group. Thus all attached Figures show mean values for 60 cells.

Results and Discussion

The scheme of the experiment permitted comparison of the effects developed by a peptide both alone and in combination with (not more than three) other peptides. Also compared was the effect of simultaneous and successive treatment with four peptides, with special regard to the possible impact of the sequence of peptides.

Since *Tetrahymena* fails to divide in culture under an oil film (Williams et al. 1980 a), maintenance of treated cells in that system enabled a long-term follow-up of receptor memory in the treated generation itself. We demonstrated earlier (Csaba et al. 1984 b) that in such cells the receptor "memory" persisted as long as nine months. Since, in view of this, no loss of memory could have occurred during the relatively short incubation times (1, 2 or 4 weeks) used in this study, the observed cellular response was due to the different hormone treatments rather than to the anaerobic conditions of maintenance.

The tested peptides had dissimilar imprinting effects on *Tetrahymena*. While insulin gave in most cases rise to a positive imprinting after one day (except when it was given alone, what possibly resulted by down-regulation of the receptor — Csaba and Kőhidai 1986), as demonstrated also earlier, TSH, oligopeptide and oxytocin failed to induce a positive imprinting in themselves and on combined simultaneous or successive application as well, and the dipeptide accounted for positive imprinting only in a single case. Nevertheless, it appears that the success or failure of imprinting was unrelated to the single or combined (simultaneous or successive) application of the peptides, and to the sequence of successive application as well. The sole exception was the dipeptide (Ala-Tyr) mentioned above (Fig. 1).

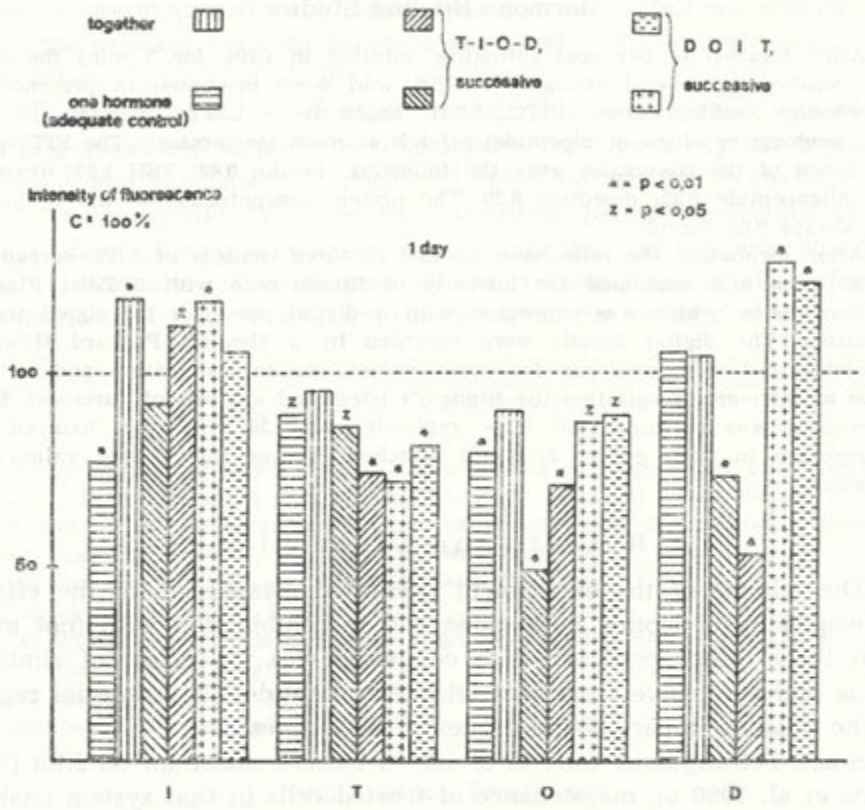

Fig. 1. Hormone binding capacity of *Tetrahymena* one day after simultaneous or successive treatment with different peptides, related to the untreated control as 100. I — insulin; T — TSH; O — oxytocin or oligopeptide; D — dipeptide. The letters in the bottom row designate the FITC-labeled hormone used for binding studies. Adequate control: treatment with only one hormone which is equal to the hormone used for binding study, too (indexed in the bottom row). T-I-O-D or D-O-I-T are the sequences of hormones given in the successive treatments

Examination of the cells maintained under the oil film for one week showed that simultaneous treatment with the four peptides was always more effective than treatment with any of these peptides in itself. In the case of large molecular weight peptides the T-I-O-D sequence (the advantage of large molecules) increased the imprinting effect related to the single treatment, while the D-O-I-T sequence (the advantage of small molecules) decreased it. The imprinting activity of the small peptides was considerably reduced when they followed after the larger ones, and when the dipeptide was applied before oxytocin. Nevertheless, it cannot be excluded that not so much these circumstances as the later treatments were responsible for suppression of the imprinting effect (Fig. 2).

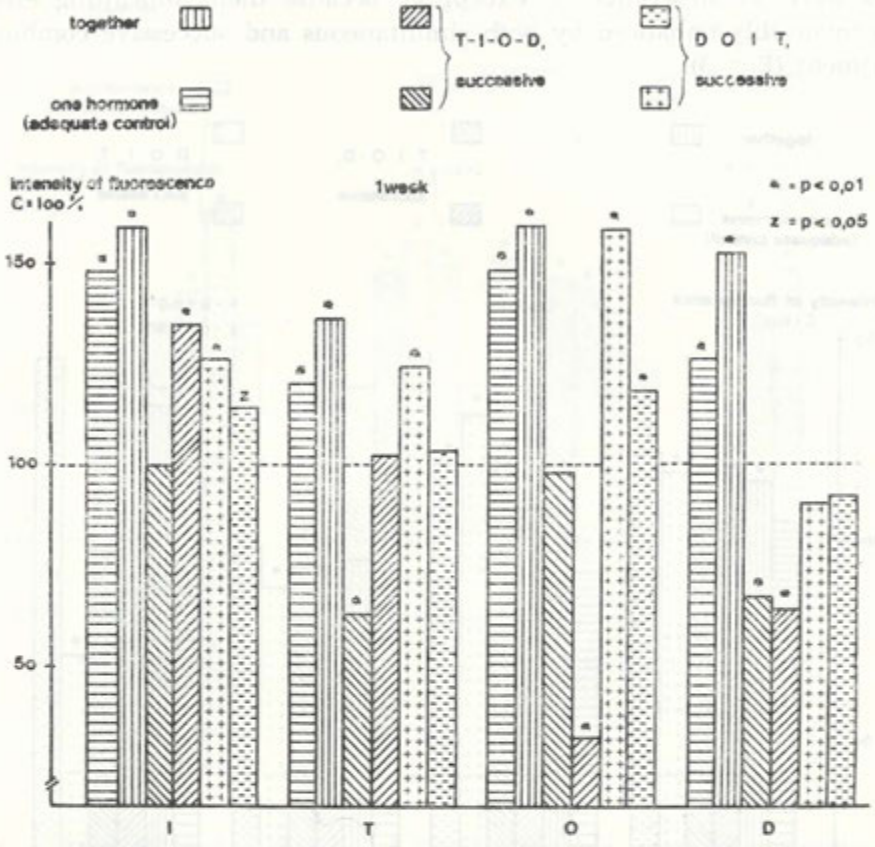

Fig. 2. Hormone binding capacity of *Tetrahymena* one week after combined simultaneous or successive treatment with different peptides, related to the untreated control as 100. Indices as in Fig. 1

Remarkably, TSH and the oligopeptides, and in most experiments also the dipeptides, which showed no measurable positive imprinting after one day, did show it powerfully after one week, and after two and four weeks as well. It follows that lack of manifestation of imprinting shortly after it had taken place does not exclude its late manifestation, which may depend on the quality or molecular weight of the imprinting molecule. It appears furthermore that such late manifestations were enhanced rather than depressed by simultaneous or successive treatment with hormone combinations.

After two weeks a certain equalization of the inter-group differences could be observed, inasmuch as the simultaneous or successive treatments with hormone combinations did not appreciable influence the development of imprinting relative to effect of the single hormones. The dipep-

tides were, at all events, an exception, because their imprinting effect was invariably enhanced by both simultaneous and successive combined treatment (Fig. 3).

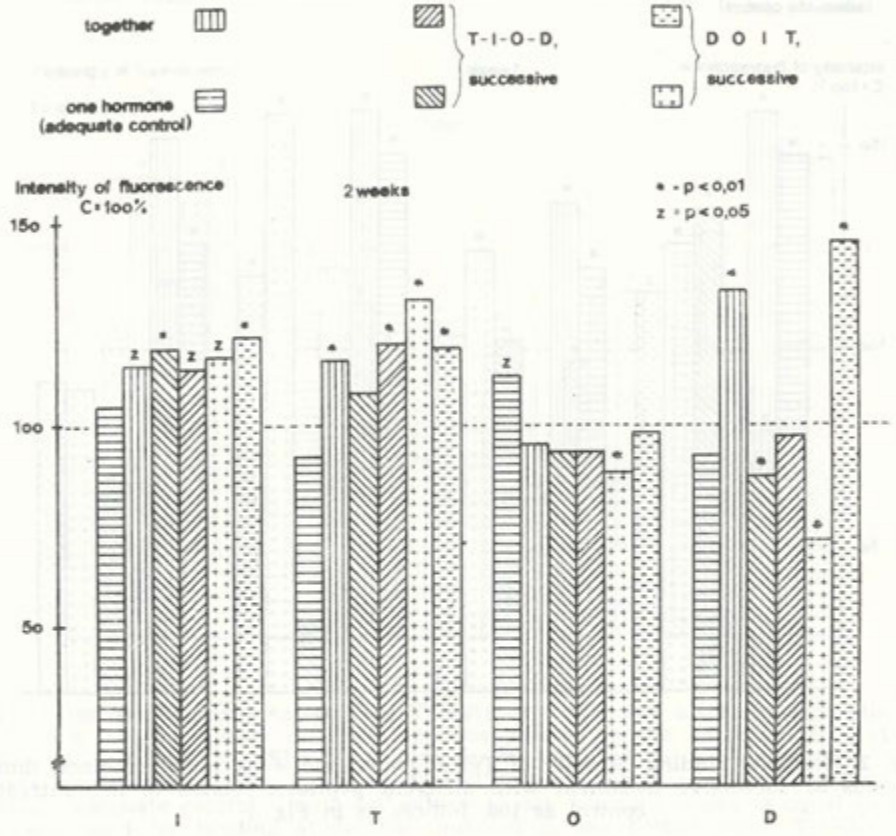

Fig. 3. Hormone binding capacity of *Tetrahymena* two weeks after simultaneous or successive treatment with different peptides, related to the untreated control as 100. Indices as in Fig. 1

After four weeks the tendencies were less unequivocal, although the effect of imprinting was still obvious. A certain equalization of intergroup differences was still present, and reversion of the peptide sequences in the successive treatment gave rise to certain differences, e.g., in the case of insulin and dipeptide (Fig. 4).

We demonstrated earlier (C s a b a et al. 1982) that *Tetrahymena* possesses a receptor "memory", which resembles to a certain extent the neuronal memory. Retroactive interference, i.e., amplification of the "memory" by reexposure to the active molecule also occurs in *Tetrahymena* (C s a b a et al. 1984 a). Although induction of a second imprinting

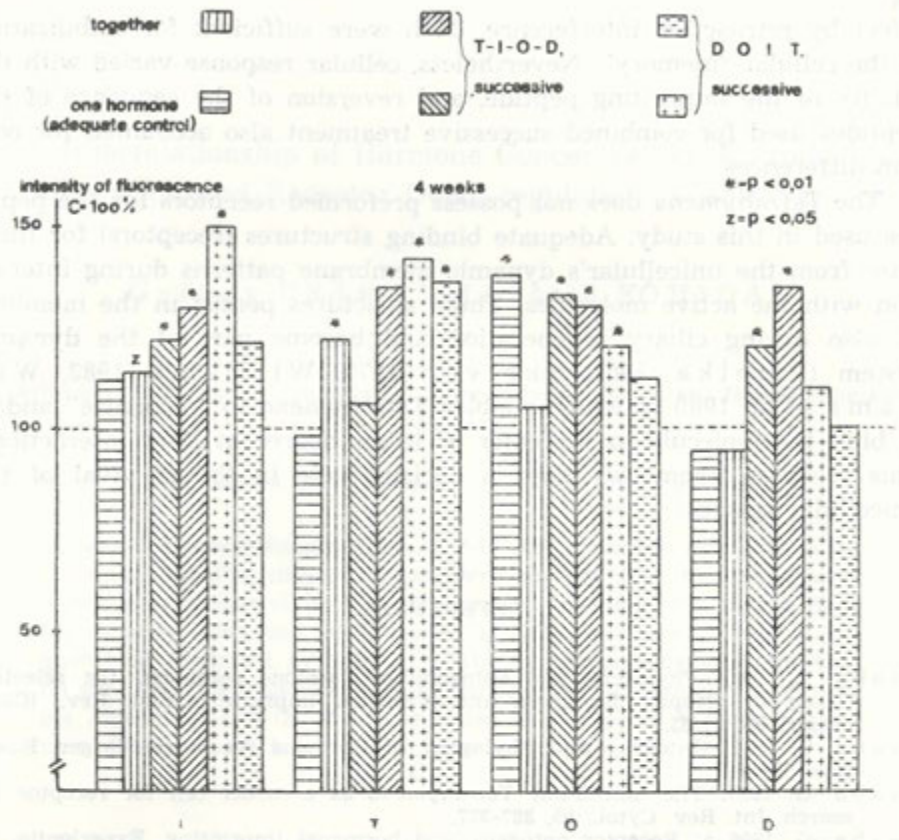

Fig. 4. Hormone binding capacity of *Tetrahymena* four weeks after simultaneous or successive treatment with different peptides, related to the untreated control as 100. Indices as in Fig. 1

immediately after the primary one hampered the establishement of the latter (Csaba et al. 1984 a), the scope of the "memory" of the *Tetrahymena* seemed to be wide enough to cover simultaneously a fair range of different active molecules. Simultaneous exposure to four peptides did not interfere with the normal course of imprinting, and seemed to enhance rather than suppress it in part of the cases. This effect was in all probability associated with the stimulation of dynamic membrane patterns (Koch et al. 1979). It ought to be mentioned in this context that *Tetrahymena* meets a great variety of active molecules also in its natural environment and should, accordingly, possess an extraordinarily dynamic membrane to meet the challenge.

Successive treatment with four peptides at 24 h intervals did not interfere with imprinting either. It follows that while exposure to a foreign peptide immediately after imprinting extinguished the latter's

effect by retroactive interference, 24 h were sufficient for stabilization of the cellular "memory". Nevertheless, cellular response varied with the quality of the imprinting peptide, and reversion of the sequence of the peptides used for combined successive treatment also accounted for certain differences.

The *Tetrahymena* does not possess preformed receptors for the peptides used in this study. Adequate binding structures (receptors) for these arise from the unicellular's dynamic membrane patterns during interaction with the active molecules. These structures persist in the membrane also during ciliary regeneration and become part of the dynamic system (Pitelka 1961, Skriver 1979, Williams 1983, Williams et al. 1980 b) which enables *Tetrahymena* to "recognize" and/or to bind the molecule to a greater or lesser degree at later interactions. This receptor "memory" plays a decisive role in the survival of the unicellular species.

REFERENCES

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

Csaba G. 1981: Ontogeny and Phylogeny of Hormone receptors. Karger, Basel-New York.

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

Csaba G. 1986 a: Receptor ontogeny and hormonal imprinting. Experientia, 42, 750-759.

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

Csaba G. and Kőhidai L. 1986: Modelling the insulin receptor in the *Tetrahymena*. Time-dependence of receptor formation, down regulation and imprinting. Acta Protozool., 25, 331-338.

Csaba G., Németh G. and Vargha P. 1982: Development and persistence of receptor "memory" in a unicellular model system. Exp. Cell. Biol., 50, 291-294.

Csaba G., Németh G. and Vargha P. 1984 a: Receptor "memory" in *Tetrahymena*: does it satisfy the general criteria of memory? Exp. Cell. Biol., 52, 320-325.

Csaba G., Darvas Zs., László V. and Vargha P. 1984 b: Influence of prolonged life span on receptor "memory" in a unicellular organism, *Tetrahymena*. Exp. Cell. Biol., 52, 211-216.

Koch A. S., Fehér J. and Lukovics I. 1979: Single model of dynamic receptor pattern generation, Biol. Cybern., 32, 125-139.

Pitelka D. L. 1961: Fine structure of the silverline systems of three tetrahymenid ciliates. J. Protozool., 8, 75-89.

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Interrelationship of Hormone Concentration, Hormonal Imprinting and Receptor Down-regulation in *Tetrahymena*

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Synopsis. Higher concentrations (10^{-8}-10^{-5}M) of insulin down regulated the insulin binding sites of *Tetrahymena* one day after treatment, however concentrations of 10^{-6}-10^{-13}M caused imprinting with higher binding capacity. After one week imprinting had been observed as a consequence of treatment by each dose. The experiments demonstrate, that (1) very low (100 femtomole) insulin concentration is enough for provoking imprinting and (2) down regulation is a response to hormone excess already at the level of *Tetrahymena*.

In higher organisms the primary interaction between the still immature, but genetically encoded receptor and the adequate hormone takes place in the perinatal period and gives rise to hormonal imprinting, which accounts for receptor amplification, i.e., for establishment of the hormone binding capacity characteristic of adulthood (Csaba 1980, 1986 a). Since unicellular organisms are, by nature, lacking genetically encoded hormone receptors, they present membrane-associated protein configurations of varied quality as hormone binding structures, and transmit these to many progeny generations (Csaba 1985, 1986 b, Csaba et al. 1982). The establishment of such receptor "memory" is being influenced by many factors, of which the concentration of the hormone was the subject of the present study.

Materials and Methods

Tetrahymena pyriformis GL cells maintained in 1% peptone plus 0.1 yeast extract containing Bacto-tryptone medium (Difco, Michigan, U.S.A.) at 28°C were treated (or not treated, as control) with different insulin (Semilente MC, Novo,

Denmark) concentrations (range: 10^{-3}-10^{-13}M) for 1 h (sample size: 10 ml, volume 4×10^5 cell/ml), returned to plain medium for 24 h, then incubated in presence of fluoresceine-isothiocyanate-labeled insulin (FITC, BDH, England) for 1 h, and assayed for intensity of fluorescence in a Zeiss Fluoval cytofluorimeter, which was connected with a HP41CX calculator, programmed for the statistical evaluation of mean values, standard deviation, and significance of inter-group variation. At three levels of treatment (10^{-13}, 10^{-8} and 10^{-3}M) the fluorescence assay was done one week later, too. Twenty cells were assayed at each level of treatment in five replica experiments, thus the values shown in Fig. 1 represent means for 100 cells.

Results and Discussion

The experimental results have demonstrated that already a very low (100 fmol) concentration of insulin was sufficient to induce a lasting imprinting, which did not diminish after transmission to as many as 70 generations within a week's time. It was also demonstrated that insulin imprinting did not become stronger with the concentration increase, and exposure to higher insulin concentrations ($10^{-3}$$10^{-5}$M) even gave rise to down-regulation within 24 h (Fig. 1). This seems logical,

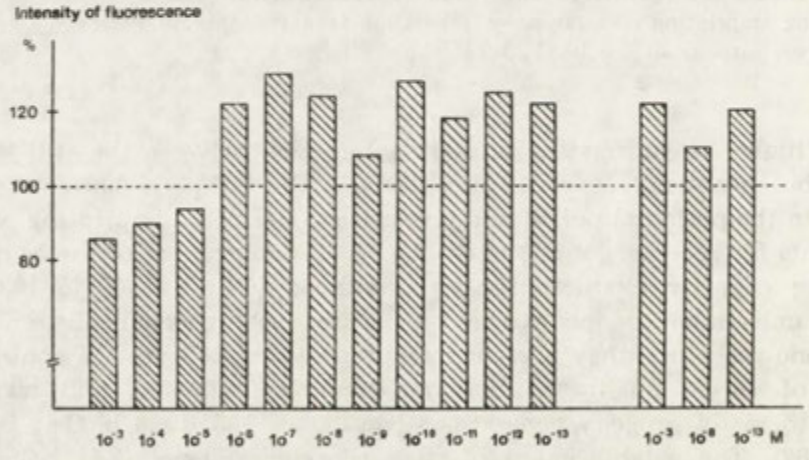

Fig. 1. Binding of insulin to *Tetrahymena* imprinted by different concentrations of insulin one day (left side) and one week (right side) after treatment, related to the control as 100%

if the appearance of coated pits and internalization — in every respect similar to those occurring in higher organisms — in *Tetrahymena* is taken into consideration (Csaba et al. 1984). The critical threshold between the imprinting and down-regulatory effects of insulin on the unicellular is to be sought within the concentration range 10^{-5}-10^{-6}M

but, after one week, the down-regulation elicited by high insulin level also transformed to an imprinting effect. Since, in view of this, imprinting was apparently independent of the applied hormone concentration, the presence, or rather the quality of the hormone seemed to play the decisive role. Since the "recognition" of, or "memory" of, useful and noxious environmental molecules is of vital importance for the survival and reproduction of a protozoon, the dependence of the imprinting mechanism on qualitative rather than quantitative factors seems to be wholly justified from the biological point of view.

Analysis of the present results from the angle of down-regulation — a mechanism which has been well established in higher organisms (Gammeltoft 1984, Gorden et al. 1980, Marshall and Olefsky 1980) — permits the conclusion that such a mechanism also operates at the unicellular level, at which it would also render the target cell refractory to excessive hormonal influence. Since down-regulation begins to operate already at the primary interaction of the target cell with the hormone if the latter is present in excess, there is reason to postulate that down-regulation is not so much an integral part of hormonal regulation as response shown to hormone excess also by "naive" — not encoded — cells after presentation of a hormone binding (receptor) structure (Csaba and Kőhidai 1986). The membrane of *Tetrahymena* being extraordinarily dynamic, exposure to insulin (or another hormone) for 1h seems to be sufficient to initiate either down-regulation or hormonal imprinting, depending on the applied concentration of the hormone.

REFERENCES

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

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

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

Csaba G. 1986 b: Receptor ontogeny and hormonal imprinting. Experientia, 42, 750-759.

Csaba G., Németh G. and Vargha P. 1982: Development and persistence of receptor "memory" in a unicellular model system. Exp. Cell. Biol., 50, 291-294.

Csaba G., Gruszczyńska M., Madarász B. and Sudár F. 1984: Coated pits and coated vesicles in unicellulars (*Tetrahymena, Critidia*) and in Hydra. Acta Morph. Hung., 32, 181-186.

Csaba G. and Kőhidai L. 1986: Modelling the insulin receptor in the *Tetrahymena*. Time dependence of receptor formation, down-regulation and imprinting. Acta Protozool., 25, 331-338.

Gammeltoft S. 1984: Insulin receptors: binding kinetics and structure-function relationship of insulin. Physiol. Rev., 64, 1321-1378.

Gorden P., Carpentier J.-L., Freychet P. and Orci L. 1980: Morphologic probes of polypeptide hormone receptor interactions. J. Histochem. Cytochem., 28, 811-817.

Marshall S. and Olefsky J. M. 1980: Effects of insulin incubation on insulin binding, glucose transport and insulin degradation by isolated rat adipocytes. J. Clin. Invest., 66, 763-772.

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First Record of Microsporan Infection on *Sitophilus oryzae* (L.) in India

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Synopsis. A species of microsporan, *Microsporidium sitophili* from a coleopteran stored-grain pest, *Sitophilus oryzae* (L.) is proposed in this communication.

Microsporans are very common parasites of insects. Some of them occur in the host's alimentary canal, some in the adipose tissue, malpighian tubules, hemocytes or muscles, and some others in the gonads. In mature insects microsporans bring about decreased fertility or even sterility of males and females. Transovarian dissemination of microsporans is of great epizootiological importance and considerably increases their usefulness in biological pest control (Lipa 1976). Further, microsporans contribute to the shortening of life and host's death is usually preceded by a drop of vigor, loss of appetite and decreased response to external stimuli. So a close study of the biology and ecology of respective species of microsporans becomes very essential.

The present communication deals with the study of microsporan infection in a common stored-grain pest — *Sitophilus oryzae* (L.) (*Coleoptera*: *Curculionidae*). The parasite damages the malpighian tubules and fat bodies of the host and possesses some distinctive characters of its own. This is the first record of a microsporan infection in *S. oryzae* in India.

Material and Methods

The insect hosts were collected from a godown at Chinsurah, W.B., India and brought alive to the laboratory for examination for protozoan parasites. Smears of the infected parts were air dried, fixed in methanol and stained in

Giemsa's solution after hydrolysing for 10 min in 1N HCl. Some of the wet smears were fixed in Schaudinn's and Carnoy's fluids and subsequently stained with Heidenhain's iron haematoxylin and PAS reagent to reveal the presence of vacuoles and polar cap respectively. For differentiation of the nucleus the method of Weiser (1976) was adopted.

Observations

Microsporidium sitophili

Host: *Sitophilus oryzae* (L.) (*Coleoptera*: *Curculionidae*).
Site of infection: Malpighian tubules and fat bodies.
Intensity of infection: 22.3% of the hosts were infected with this parasite.
Type slide: In slide No. SM/1, Protozoology Laboratory, Department of Zoology, University of Kalyani, W.B., India.
Spore: The spores are ellipsoidal in outline (Fig. 1 a, Pl. I a) measuring 3.32 μm to 4.98 μm (mean 4.14 μm and standard deviation 0.659) in length and 1.32 μm to 2.5 μm (mean 1.68 μm, standard deviation 0.312) in width. Each spore is surrounded by two walls — the outer exospore and the inner endospore. At the anterior and posterior ends of the spore, the polaroplast and posterior vesicle are observed after staining with Heidenhain's haematoxylin (Fig. 1 b). When stained with Giemsa's solution following hydrolysis in 1N HCl, the sporoplasm is seen to extend in between the polaroplast and posterior vesicle (Fig. 1 c). A PAS-positive polar cap is seen at the anterior end of the spore in PAS stained preparations (Fig. 1 d). Staining with Weiser's technique shows one round nucleus located centrally (Fig. 1 e, Pl. I b). The polar filament is uniformly thin and measures 20-28 μm (Fig. 1 f). The pansporoblasts with pansporoblastic membranes are irregularly rounded measuring 32.0 μm in diameter each containing 35 to 50 spores (Fig. 1 g, Pl. I c). The frequency distribution of the size of the spores in several dimensionable groups are given in Table 1.

Table 1

Frequency distribution of the size of one sample of 70 spores of *Microsporidium sitophili*

Sample	Dimensionable groups in microns				
	Length		Width		
	3.32–4.15	4.2–4.98	1.32–1.66	1.67–1.82	1.83–2.0
Fixed and stained spores	31	39	42	3	25

Schizont: Schizonts of different sizes measuring 15-20 μm with variable number of nuclei were observed. The largest schizont contained 12 nuclei and measured 20 μm in diameter (Fig. 1 h, Pl. I d).
Sporont: These are spherical to oval in shape with variable numbers of nuclei. The largest sporont observed measured 25.0 μm to 30.0 μm in diameter with 40 nuclei (Fig. 1). The nuclear membrane has not been observed.

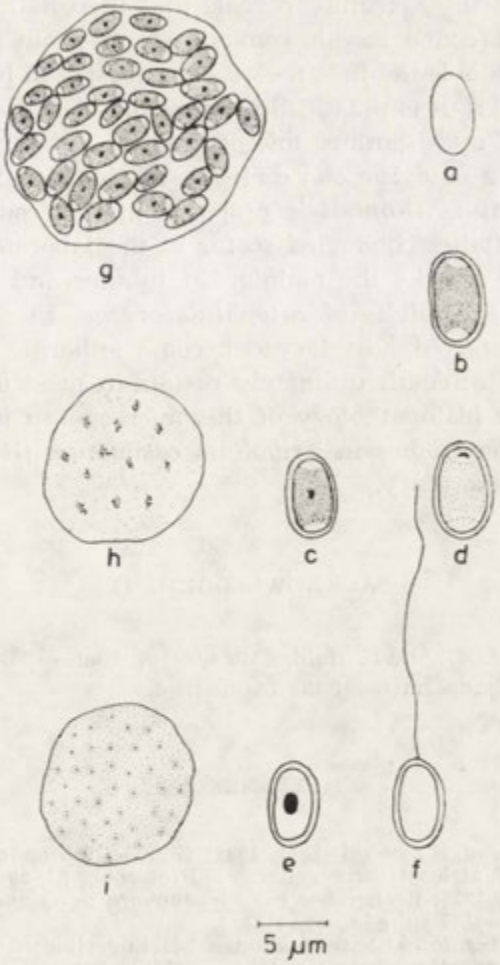

Fig. 1 a-i. Camera lucida drawings of different stages of life cycle of *Microsporidium sitophili*, a — a fresh spore, b — a spore stained by Heidenhain's haematoxylin, c — a spore stained by the Giemsa's stain, d — a spore stained according to the PAS technique, e — a spore stained with Weiser's technique for localization of nucleus, f — a spore with polar filament fully released, g — a pansporoblasts showing about 40 spores, h — a schizont showing 12 nuslei, i — a sporont showing 40 nuclei

Discussion

As the several spores are produced from each sporont during its sporogony the present form can be placed tentatively under the genus *Pleistophora* Gurley, 1893. The present form exhibits resemblances with *P. melolonthae* Krieg, 1955 and *P. periplanetae* Georgevitch, 1927 since the size of the spores is similar in these three forms. Otherwise, it is distinctly different from the above-mentioned species in other features.

A perusal into the literature reveals that there is no previous record of microsporan infection in this common stored-grain pest — *Sitophilus oryzae*. As such, this is the first record of microsporan infection in *S. oryzae*. However, today it is practically impossible to make a correct generic assignment of a multisporans microsporidian without electron microscopy (Canning and Hazard 1982). In this situation the collective name *Microsporidium sitophili* is proposed in this communication.

Preliminary studies conducted so far in the laboratory have revealed that the parasite attacks the malpighian tubules and the fat bodies of both the larvae and adults of *Sitophilus oryzae*. In the case of severe infestation the infected host larvae become lethargic and show a tendency to aggregate; death ultimately results in most of the larvae. Observations on the histopathology of this microsporan in its natural host and cross-infection studies in *Tribolium castaneum* (Herbst) are now in progress in the laboratory.

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REFERENCES

Canning E. U. and Hazard E. I. 1982: Genus *Pleistophora* Gurley, 1893: An assemblage of at least three genera. J. Protozool., 29, 39-49.

Georgevitch J. 1927: Recherches sur *Pleistophora periplanetae* Lutz and Splendore. Arch. Zool. Exp. gén., 66, 1-21.

Krieg A. 1955: Über infektionskrankheiten bei Engerlingen von *Melolontha* spec. unter besonderer Berücksichtigung einer Mikrosporidien-Erkran-Kung. Zentr. Bakt., II, 108, 535-538.

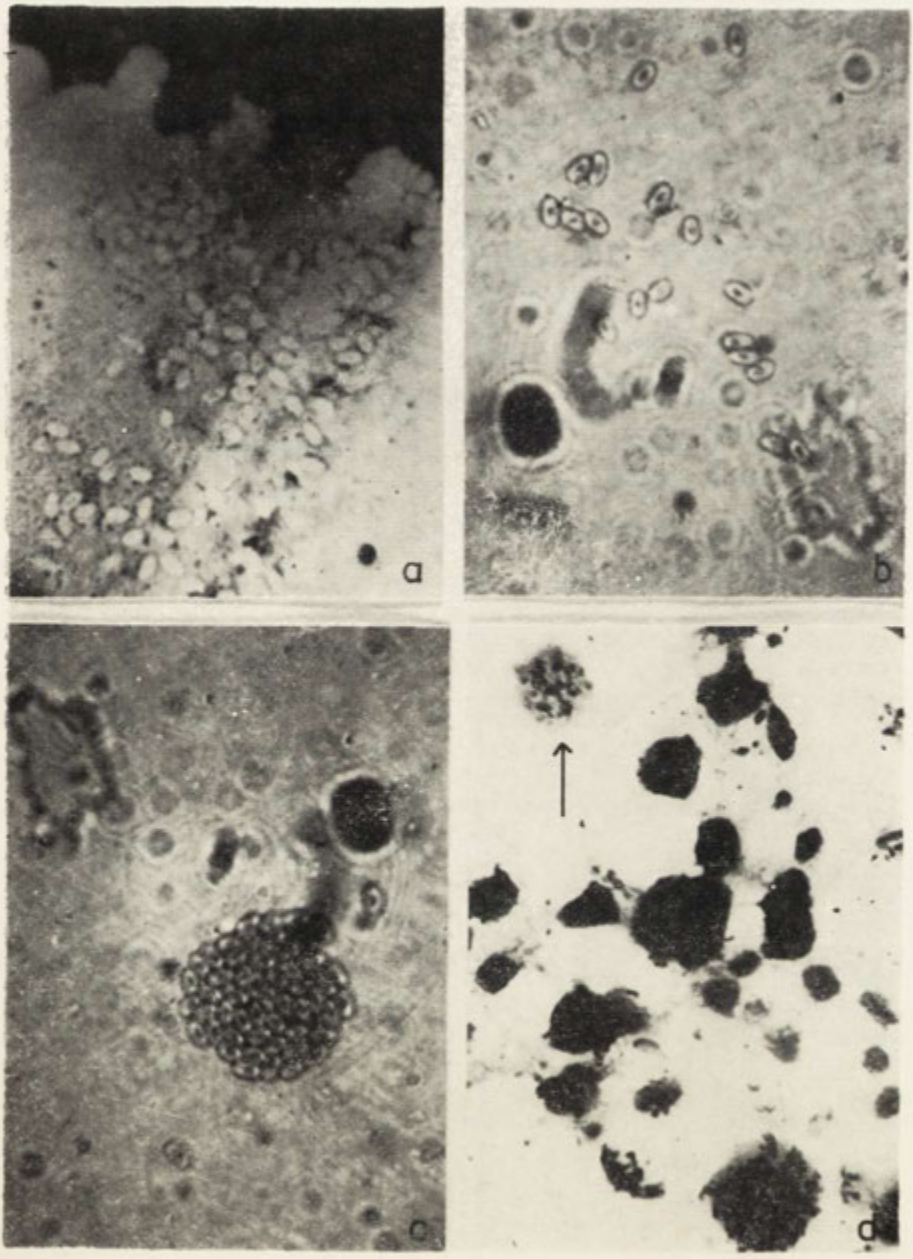
Lipa J. J. 1976: Protozoa as the regulators of pest insects populations. Wiad. Parazytol., 22, 365-368.

Weiser J. 1976: Staining of the nuclei of microsporidian spores. J. Invertebr. Pathol., 28, 147-149.

EXPLANATION OF PLATE I

Photomicrographs of *Microsporidium sitophili*

a: Showing ellipsoidal spores
b: Spores stained by Weiser's technique
c: Spores of *Microsporidium sitophili*, in pansporoblasts
d: Schizonts of *Microsporidium sitophili*



S. Ghose auctor phot.

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A Simple Method for Production of Amicronucleate Cell Lines in Ciliates by cis.-Dichlorodiammineplatinum II

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Synopsis. Treatment with cis.-Dichlorodiammineplatinum II (cis. platin), a DNA cross-linking agent (Roberts and Pascoe 1972) induces amicronuclearity in widely divergent species of ciliates. Cells of *Blepharisma musculus*, *Pseudourostyla levis*, *Frontonia leucas* and *Spirostomum ambriguum* possess minute and multiple micronuclei. Following treatment with cis. platin, micronuclei are affected specifically resulting in their non-propagation in the clonal progeny. The yield of amicronucleate cells produced with a specific dose of cis. platin treatment varies among different ciliates, being the highest in *Blepharisma* and the lowest in *Frontonia*. Viable amicronucleate clones have been successfuly raised from all the four ciliate species by this method.

The unicellular ciliated protozoa are distinguished by nuclear dimorphism. The macronucleus is essential for cellular survival and propagation, while the micronucleus acts as the germinal reservoir and reorganizes the nuclear apparatus during sexual reproduction. However, there is no definite consensus about the exact role played by the micronucleus in the somatic cellular functions. One of the ways to probe this question is to obtain amicronucleate cell lines and study the consequences of amicronuclearity on vegetative functions. Occurrence of amicronucleate cell lines in nature is a very rare phenomenon. Therefore, such investigations have to be conducted on amicronucleate cells produced in the laboratory through experimental methods. Mostly those species with one to two large micronuclei have been studied as these micronulei can be eliminated from the cell by micromanipulation or through microbeam irradiation (Ng 1986).

Due to technical and manipulative difficulties, species which possess multiple and minute micronuclei have not been investigated for such

studies. In the present study, we report a convenient and reliable method by which cells can be amicronucleated by treatment with a DNA binding agent viz. cis.-Dichlorodiammineplatinum II (cis. platin) (Rosenberg et al. 1965, Howle and Gale 1970, Roberts and Thomson 1979) irrespective of the number and size of micronuclei in the cell.

Material and Methods

Cells of four different species of ciliates were studied in the present work. *Blepharisma musculus* and *Pseudourostyla levis* were cultured in modified Pringsheim's medium (Chapman-Andersen 1958) and fed daily with axenically grown green alga *Chlorogonium elongatum* (Ammermann et al. 1974). *Frontonia leucas* and *Spirostomum ambiguum* were grown in Pringsheim's culture medium supplemented with boiled rice grain or pieces of cabbage leaf for supporting mixed bacterial growth. All experimental and routine cultures were kept at $23 \pm 1°C$ in temperature regulated BOD chambers.

For calculation of the cell length and width, fifty cells of each ciliate species in the G_1 phase were immobilized in 5% methyl cellulose and measurements of live cells taken. A synchronous population of G_1 cells was obtained by isolating with a micropipette, late dividing cells from the mass culture under a stereo microscope. Newly divided cells were allowed to grow for one hour in the G_1 phase prior to the measurement and subsequent experiments. The micronuclear size of G_1 cells was calculated by measuring fifty micronuclei of each ciliate species from the Feulgen stained preparations. All measurements were done with an ocular micrometer (Leitz).

Preparation of Cis. Platin Solution and Treatment

Cis. platin (Sigma, mol. wt. 300.1, anhydrous) is slightly soluble in water (0.253 g/100 ml at 25°C) (Kauffman 1963). It is stored in dark in the crystalline state. A fresh stock solution of the chemical (1500 µg/ml) was prepared in 0.9% saline prior to use. To ensure maximal solubilization, the solution was forced in and out of a hypodermic syringe (needle No. 20) several times at room temperature. Stock solution was diluted serially with the culture medium. After trials with various dosages, a concentration of 150 µg/ml for 120 min was chosen as it permitted fifty per cent or more survival of treated cells in the various species tested.

For treatment with cis. platin, cells in the G_1 phase were used. In each experiment, three replicates of one hundred cells each were treated with the chemical. After treatment, cells were washed several times with the culture medium.

Results and Discussion

All the four aforementioned ciliate species are of different sizes and possess multiple micronuclei of minute sizes (Table 1 and Pl. I-II). In all cases, after treatment with cis. platin, cells show unidisturbed nuclear

Table 1

Cell sizes and micronuclear sizes and number in different ciliate species

Ciliate species	Size of the Cell		Number of micronuclei (range)	Size of the micronucleus (diameter) (μm±S.E)
	Length (μm±S.E)	Width (maximum) (μm±S.E)		
Blepharisma musculus	142,50±1.25	38.29±1.50	8–12	1.66±0.03
Pseudourostyla levis	275.75±1.50	72.15±2.25	6–9	4.14±0.23
Frontonia leucas	300.85±1.80	146.50±2.15	5–8	3.05±0.19
Spirostomum ambiguum	580.55±1.70	62.90±2.50	9–12	1.86±0.02

Values denote mean of fifty readings with standard error (±).

cytology, generation time and cellular morphology for one to two division cycles, which is termed as the 'lag-period'. Afterwards, amicronucleate cells (Pl. I-II) begin to arise in the descendant progeny due to the failure of micronuclear division, which normally should occur concomitant with the cellular fission. However, few cells are not affected and continue to retain their micronuclei.

Dividing cells were isolated at random from the treated cultures for raising amicronucleate clones. After the cell division, one of the sister cells was stained with the Schiff's reagent and the other allowed to multiply. If a particular sister cell was found to be amicronucleate, then the amicronucleate clonal line was established from the other sister cell. The proportion of amicronucleate cells in cultures gradualy increases, as they proliferate to produce more of their own progeny till the number reaches a peak. The maximum number of amicronucleate cells obtained with a specific treatment, varies from species to species. The maximum yield depends upon the initial proportion of amicronucleate cells, generation time of amicronucleate cells and their viability. In this respect, the maximum proportion of amicronucleate cells are noticed in *Blepharisma* cultures, and the minimum in *Frontonia* cultures (Table 2). No significant correlation could be observed between the micronuclear size and the proportion of amicronucleate cells (Table 1, 2). So, the micronuclear size perhaps does not play an important role in its sensitivity towards cis. platin. One may suggest that the differential micronuclear sensitivity of ciliates probably reflects upon the different nature of genomic organization in various ciliates.

Amicronucleate cells in the early stages are easily recognized by comparatively dark and rounded appearance, together with various vegetative disfunctions such as slow locomotion, aberrant cytokinesis, prolonged generation time and low viability (details in preparation). The

Table 2

Quantitative analysis of certain cellular events following cis. platin treatment in different ciliate species (Dosage: 150 μg/ml for 120 min)

Ciliate species	Maximum proportion of amicronucleated cells (%)±S.E.	Maximum proportion of amicronucleated cells with distorted macronuclear cytology (%)±S.E.	Survivability of amicronucleated cells (%)±S.E.	Mean generation time ±S.E. (hours)		t* values	Time taken by amicronucleated cells to offset vegetative dysfunctions (days) ±S.E.
				micronucleate cells	amicronucleated cells		
Blepharisma musculus	82±0.70	80±0.60	18±0.50	16±0.14	36±0.42	60.60	72±1.80
Spirostomum ambiguum	78±0.90	75±0.80	38±0.70	32±0.33	36±1.72	7.10	18±1.65
Pseudourostyla levis	75±1.70	73±1.50	28±0.40	19±0.14	33±0.23	5.14	33±1.65
Frontonia leucas	58±1.20	55±1.30	35±0.55	24±0.31	32±1.66	12.80	60±1.85

*$p < 0.001$ (highly significant) for all the species.
Number of experiments = 3
In each experiment, 50 cells were scored (n = 150 cells)

macronuclear cytomorphology is also distorted. All amicronucleate cells are not viable. Nonviable cells do not multiply further and cytolyse. The viability index as measured by the ability of amicronucleate cells to establish healthy amicronucleate clones is characteristic of the species (Table 2). Viable amicronucleate cells pass through a long period of vegetative disfunctions that ranges from eighteen to seventy two days in different species, before they acquire normal morphology, macronuclear cytology and generation time (Table 2). The stabilized viable amicronucleate cell lines continue proliferating to produce amicronucleate progeny.

Earlier, several investigators have used successfully chemicals for amicronucleation purposes. These include colchicine in *Paramecium aurelia* (Butzel 1953) and *P. caudatum* (Mikami 1979 b) urea in *P. caudatum* (Miyake 1955) and hydroxyurea in *Euplotes aediculatus* (Kloetzel 1980). The yield and survivability of amicronucleate cells obtained by the above methods have not been reported. The technique of amicronucleation by physical methods viz. micromanipulation and selective irradiation (UV, X-ray and laser microbeam) have been also in use (Wells 1961, Ammermann 1970, Fokin and Ossipov 1975, Fokin 1978, Fujishima and Hiwatashi 1978, Mikami 1979 a, Kloetzel 1980, Ng 1980, Fokin and Ossipov 1981, Fujishima and Watanabe 1981, Ng 1981, Mikami et al. 1983, 1985, Ng 1986). However, only a few species (*Euplotes, Paramecium, Stylonychia* and *Tetrahymena*) with one to two large micronuclei are amenable towards these methods, particularly when micromanipulation is employed. In contrast to the above methods, the present study has shown that ciliates with minute (1.66-4.14 μm in diameter) and multiple micronuclei (up to 12) can be easily amicronucleated and viable amicronucleate clones can be raised.

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REFERENCES

Ammermann D. 1970: The micronucleus of the ciliate *Stylonychia mytilus*, its nucleic acid synthesis and its function. Exp. Cell Res., 61, 6-12.

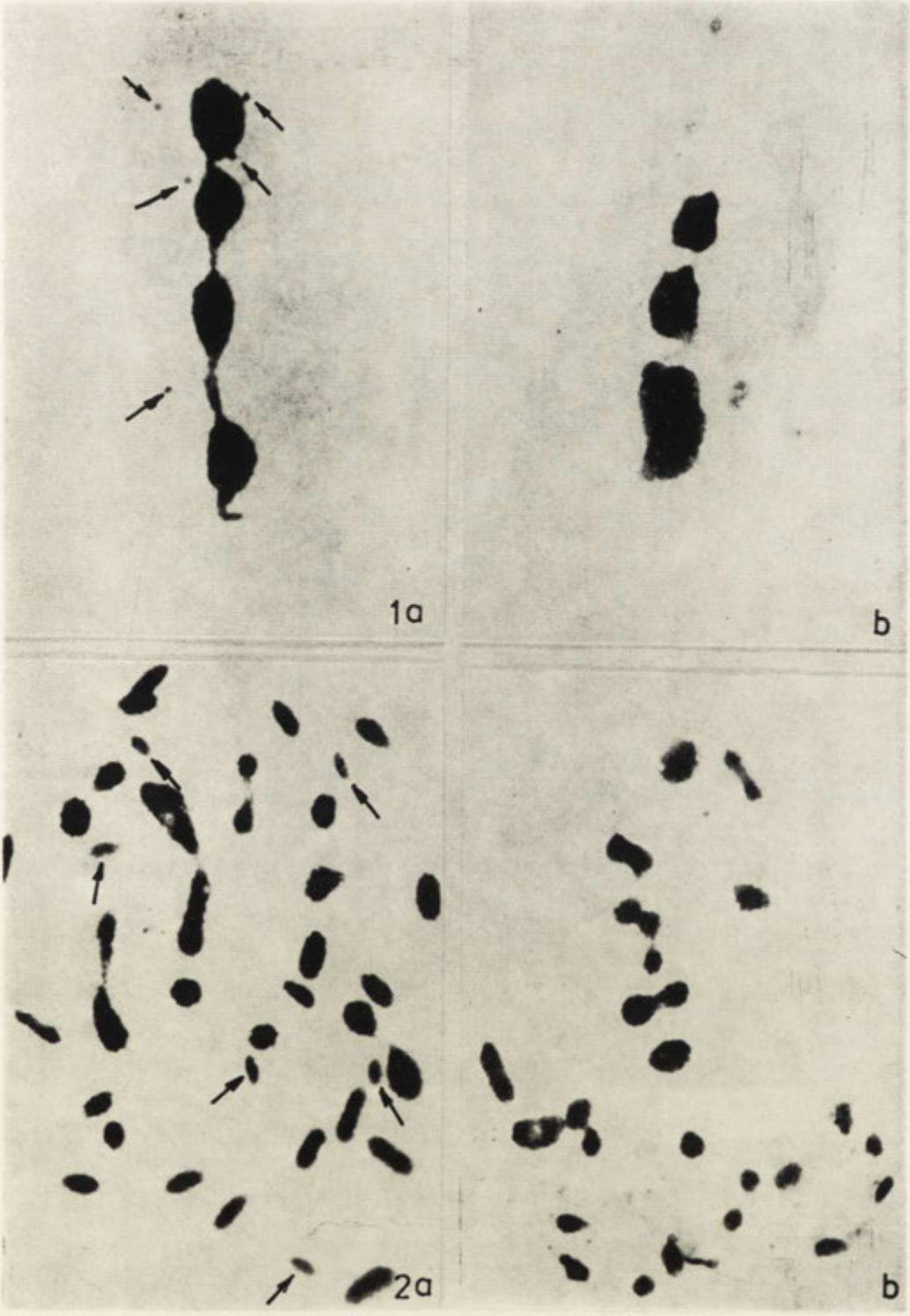
Ammermann D., Steinbrück G., Von Berger L. and Hennig W. 1974: The development of the macronucleus in the ciliated protozoan *Stylonychia mytilus*. Chromosoma, 45, 401-429.

Butzel H. M., Jr. 1953: Production of amicronucleate lines of *Paramecium aurelia* by colchicine treatment. Microb. Genet. Bull., 7, 32.

Chapman-Andersen C. 1958: Pinocytosis of inorganic salts by *Amoeba proteus*. C. R. Trav. Lab. Carlsberg, 31, 77-92.
Fokin S. I. and Ossipov D. V. 1975: Vlijanie lokal'nogo UF mikroblučenija na jadernyj apparat i citoplazm infuzorii *Paramecium caudatum*. Citologija, 17, 1073-1080.
Fokin S. I. 1978: Vlijanie lokal'nogo UF oblučenija generativnogo jadra na vegetativnye funkcii infuzorij *Paramecium putrinum*. Citologija, 20, 1426-1430.
Fokin S. I. and Ossipov D. V. 1981: Generative nucleus control over cell vegetative functions in *Paramecium*. Acta Protozool., 20, 51-73.
Fujishima M. and Hiwatashi K. 1978: Transplantation of the germ nucleus in *Paramecium caudatum*. I. Nuclei in the premeiotic S phase can enter into mitotic cycles. Exp. Cell Res., 111, 468-471.
Fujishima M. and Watanabe T. 1981: Transplantation of germ nuclei *in Paramecium caudaum*. III. Role of germinal microucleus in vegetative growth. Exp. Cell Res., 132, 47-56.
Howle J. A. and Gale G. R. 1970: cis.-Dichlorodiammine pt (II) persistent and selective inhibition of DNA synthesis *in vivo*. Biochem. Pharmacol., 19, 2757-2762.
Kauffman G. B. 1963: cis.-and trans.-Dichlorodiammineplatinum II. In: Inorganic Synthesis (ed. Kleinberg J.). McGraw Hill Book, New York 7, 239-245.
Kloetzel J. A. 1980: Further evidence for an essential role of the micronucleus in vegetative growth of the hypotrich ciliate *Euplotes*. Trans. Am. Microsc. Soc., 99, 230-231.
Mikami K. 1979 a: Stomatogenesis during sexual and asexual reproduction in an amicronucleate strain of *Paramecium caudatum*. J. Exp. Zool., 208, 121-128.
Mikami K. 1979 b: Colchicine and differentiation of macronuclear anlagen in *Paramecium caudatum*. Arch. Protistenk., 121, 213-222.
Mikami K., Kuhlmann H. W. and Heckmann K. 1983: Amicronucleate cells in *Euplotes octocarinatus* (Abstr.) J. Protozool., 30, 43A.
Mikami K., Kuhlmann H. W. and Heckmann K. 1985: Is the initiation of macronuclear DNA synthesis in *Euplotes* dependent on micronuclear functions? Exp. Cell Res., 161, 445-459.
Miyake A. 1955: The effect of urea on binary fission in *Paramecium caudatum*. J. Inst. Polytech. Osaka City Univ., Ser. D,6, 43-53.
Ng S. F. 1980: Elimination of the germ nuclei in *Paramecium tetraurelia* by laser microbeam irradiation. Exp. Cell Res., 127, 475-478.
Ng S. F. 1981: Transplantation of the germ nucleus in *Paramecium tetraurelia* and its genetic consequences. Protistologica, 17, 489-496.
Ng S. F. 1986: The somatic function of the micronucleus of ciliated protozoa. Progress in Protistology, 1, 215-286.
Roberts J. J. and Pascoe J. M. 1972: Cross linking of complementary strands of DNA in mammalian cells by antitumor platinum compounds. Nature, 235, 282-284.
Roberts J. J. and Thomson A. J. 1979: The mechanism of action of antitumor platinum compounds. Prog. Nucleic Acid Res. Mol. Biol., 22, 71-133.
Rosenberg B., Van Camp L. and Krigas T. 1965: Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature, 205, 698-699.
Wells C. 1961: Evidence for micronuclear function during vegetative growth and reproduction of the ciliate *Tetrahymena pyriformis*. J. Protozool., 8, 284-290.

EXPLANATION OF PLATES I-II

Photomicrographs of nuclear cytology of an untreated normal cell (a) with several micronuclei (arrows) and cis. platin treated cell showing amicronuclearity (b) in different ciliate species. (Feulgen).
1 a, b: *Blepharisma musculus* (× 460)
2 a, b: *Pseudourostyla levis* (× 460)
3 a, b: *Frontonia leucas* (× 460)
4 a, b: *Spirostomum ambiguum* (× 290)



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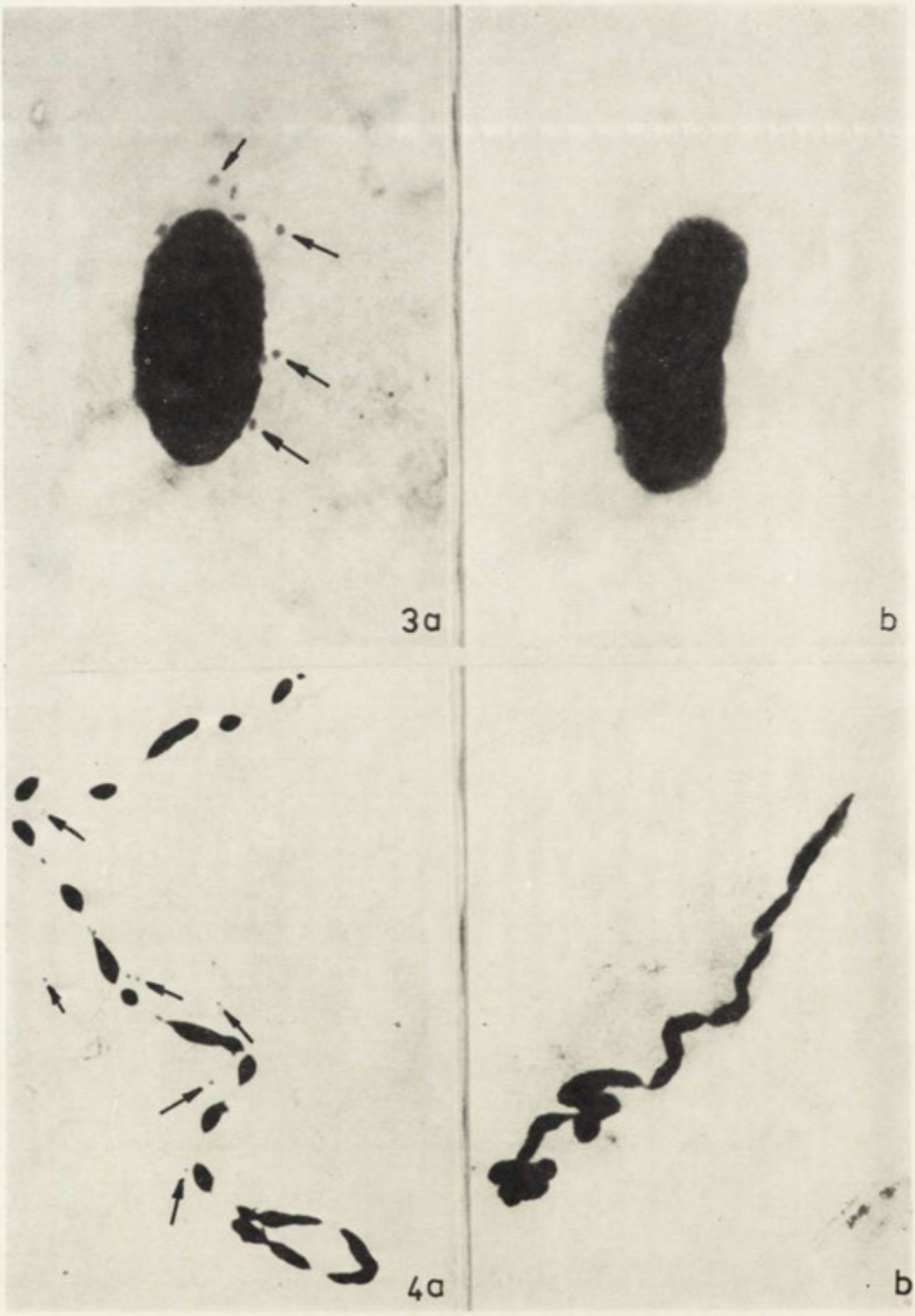

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Polish section, society of Protozoologists meetings affiliated by section

The Meeting of Protozoological Section of Polish Zoological Society was held on September 17-19, 1987 in Szczecin and was organized by Prof. Leszek Kuźnicki.

The programme of Meeting included twenty 15-30 min lasting lectures, which were presented during four sessions. A separate Poster Session was followed by the final discussion.

Abstracts of this Meeting are designated by *.

The Meeting of Protoparasitological Commission of Polish Parasitological Society was held on September 24-26, 1987 in Katowice and was organized by Prof. Stanisław L. Kazubski.

The programme of Meeting included 3 lectures and 19 abstracts, read by title. The selected abstracts were included in present material and designated by **.

All abstracts were arranged in alphabethical order.

1 *. Changes in Respiration of *Physarum polycephalum* Microplasmodia in Course of Starvation, Z. BARANOWSKI, B. HREBENDA, M. CIEŚLAWSKA and S. J. BEYLINA [1], Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland; Institute of Biophysics, USSR Academy of Sciences, Pushchino, USSR [1].

Tensiometric measurements have suggested the existence of the alternative pathway of the electron transport in the mitochondria of *Physarum polycephalum*. Now we prove this hypothesis by direct registration of O_2 uptake by *Physarum* microplasmodia. In higher plants and some microorganisms the alternative pathway is KCN-resistant but salicylohydroxamic acid (SHAM) sensitive. The same properties of respiration are observed in microplasmodia. During starvation, the KCN-resistant respiration of microplasmodia varies between 30-70% of the initial rate of oxygen consumption. The residual respiration in the presence of KCN is completely inhibited by SHAM. The kinetics of the changes of the respiration, in response to KCN, allows to deduce that the KCN resistant respiration represents the activity of the alternative pathway of the electron transport localized in the mitochondria of microplasmodia. Although the significance of the alternative respiratory pathway remains still not fully elucidated we are inclined to suggest that it represents the ability of the organism to improve its energetic conditions in any critical situations.

2 *. Small Free-living Amoebas as Hosts for *Sarcobium cytoliticum* — an Intracellular Bacterial Parasite. Wincenty DROŻAŃSKI, Department

of General Microbiology, Maria Curie-Skłodowska University, 19 Akademicka str., 20-033 Lublin, Poland.

A new bacterium fatally infecting amoebae was isolated from a raw amoeba culture freshly isolated from soil and recently described as *Sarcobium cytolyticum* gen. nov., sp. nov. Two membered amoeba-parasite culture was established, however, amoeba free medium for the growth of *S. cytolyticum* have not as yet been elaborated. Although free-living amoebae have the capacity to degrade ingested bacteria, their killing and digestion activity are incompatible with the survival and multiplication of *Sarcobium*. Electron microscopic observations have shown that both living and dead bacteria were readily phagocytized by amoebae and that their immediate residance is within endosomes. Evidences have been presented that the neighbouring phagosomes fuse extensively with endosomes containing parasites. Further on infection living bacteria were able to escape from parasitophorous vacuoles into the cytoplasm where they proliferated. The final effect of the infection was total lysis of the host cells. Yields of about 3000 bacteria per amoeba cell were obtained. It is the bacterial cell wall which is first in contact with the external environment and in the case of infectious bacteria protect the cell from the action of host's mechanisms of defence. Results are provided that the attribute of resistance of *S. cytolyticum* to the host's bacteriolytic enzymes are at least in part related to the presence of N-unsubstituted glucosamine residues in the polysaccharide chains of the cell wall peptidoglycan.

3*. Action of *Acanthamoeba castellanii* Enzymes on Lipopolysaccharide of *Salmonella minnesota* R4. Wincenty DROŻAŃSKI and Otto LUDERITZ, Department of General Microbiology, Maria Curie-Skłodowska University, 19 Akademicka str., 20-033 Lublin, Poland and Max-Planck-Institut für Immunobiologie, D-7800 Freiburg im Breisgau, F.R.G.

Five different molecular forms of fatty acyl esterases were found to be produced by *Acanthamoeba castellanii*. Enzymes present in the homogenates of amoeba cells were separated by ion-exchange chromatography on DEAE Sephadex A50 and gel-filtration on Sephadex G100. All enzymes exhibited maximal activity in the acidic environment. Three of the five esterases showed strong activity towards lipopolysaccharide of *S. minnesota*. The activity of esterases was stimulated by the presence of Triton X100 in the reaction mixture. Esterases I a and II a quantitatively remove all ester bound fatty acids, however, different initial velocities and pH optima for each fatty acid were noted. Esterase III liberated fatty acids from their acyloxyacyl linkages but was inactive towards beta hydroxytetradecanoic acid bound to the hydroxyl groups

of glucosaminyl-beta-1,6-glucosamine, hence esterase III is acyl-D-3-oxyacyl hydrolase. Evidence was provided that amoeba produces also a new class of fatty-acyl amidase. The enzyme hydrolysed the linkage between beta hydroxytetradecanoic acid and amino group of non reducing glucosamine of the lipid A backbone in the presence of long chain fatty acid bound to the NH_2 group of the reducing end of the disaccharide and hence shows to be different from fatty acyl amidase I and II of *Dictiostelium discoideum*, described by Verret et al. (J. Biol. Chem. 1982, 257, 10228).

4 *. Relationship Between Spacing of Kinetosomes and Interkinetal Distance in Somatic Domain of a Ciliate, *Dileptus*. J. DRZEWIŃSKA, Department of Cell Biology, M. Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

Dileptus has its locomotor cilia patterned into longitudinal rows, termed the kinetics. The longest fibers associated with the proximal end of kinetosome are the postciliary fibres. They arise near posterior right side of kinetosome, and extend under cell surface in right-posterior direction until they reach the margin of adjacent kinety. These fibers in *Dileptus* represent microtubular skeleton of interkinetal territory. Relationship between spacing of kinetosomes in a kinety and interkinetal distance on the right side of this kinety has been measured in four different stocks of dileptuses. Spacing of kinetosomes for the same interkinetal distance was found to be significantly different in four studied stocks. This implies the existence of stock-specific length of postciliary fibres in *Dileptus*. The spacing of kinetosomes was, however, highly correlated with the interkinetal distance in all stocks jointly, and for each stock separately. This indicates that the wider is the interkinetal distance, the more postciliary fibres it contains. Thus the enlargement of interkinetal distance during cell growth should evoke special proliferation of ciliary units, as necessary to ensure enough skeletal elements for the surface of interkinetal territories.

5 **. The Investigation *in vitro* the Rising Resistance to Imidazole Derivatives of Several *Trichomonas vaginalis* Strains. M. DYMON, K. ZEMBUROWA, E. ZIĘBA, Institute of Microbiology, N. Copernicus Academy of Medicine, 18 Czysta str., 31-121 Kraków, Poland.

Among the tested 30 *T. vaginalis* strains, in subsequent experiment, 10 were used which survived and multiplied in the same way in media with drug in dose 0.05 mg/ml and without it. The selected strains were submitted to drugs in successive, 10 passages. The drugs were incorporated into media in doses depending on their adapting capacity. The doses of drugs were the following — for metronidazole and tinidazole:

0.05, 0.1, 0.5, 1, 2.5, 5, and for clothrimazole: 5, 10, 25, 50, 100, 250 (mg/ml). Next 30 passages were done without drugs and then with drugs in subthreshold doses (for metronidazole and tinidazole 1 and 2.5, for clothrimazole 100), and correspondingly threshold doses (adequate 5 and 250 mg/ml). The results showed that the *T. vaginalis* strains kept in different degree the ability to survive and multiply when subjected to growing concentration of investigated drugs. The reduced sensitiveness towards of drugs obtained during 10 passages returns in the majority of strains to the level of initial doses (0.05 and 0.1) after 30 passages in media without drug. The results suggest that the rising of drug resistance in *T. vaginalis* strains to imidazole derivatives is possible, but in our experiments this process had only an adapting character, therefore was conditioned by phenotypical changes.

6 *. The Influence of Liposomes Containing Cholesterol on Phagocytic Activity and Proliferation of *Tetrahymena* Cells. H. FABCZAK, Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur str., 02-093 Warszawa, Poland.

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

7 *. The Resting and Action Membrane Potentials of Ciliate *Blepharisma japonicum*. S. FABCZAK and H. FABCZAK, Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur str., 02-093 Warszawa, Poland.

The electrophysiological studies were performed on a fresh water

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

8 *. The Hyalosphere, a Model to Investigating *in vivo* Membrane-cortex Interactions in Amoeboid Movements and Endocytosis in *Amoeba proteus*, Andrzej GRĘBECKI, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

Amoebae heated to 40 ± 5°C for 15-30 min become undifferentiated motionless spheres. Subsequently, at the room temperature, a circular contraction fractionates their contents into the granuloplasm aggregated at the cell centre, and the hyaloplasm forming a 50 μm wide ring around the periphery. Such cells (the hyalospheres) manifest *in vivo* the dynamics of membrane-cortex contacts, which in untreated amoebae are supposed to be responsible of some locomotory and endocytotic movements. In many hyalospheres consecutive optically dense sheets of peripheral hyaloplasm are, every 5-10 s, serially detached from the cell membrane and retracted inward to the hyalo-granuloplasm border. This phenomenon may serve as a model of frontal activity of normal locomoting amoebae, since in their fronts the contractile MF layer is found (in EM) to be disengaged from the membrane and retracted behind the hyaline cap. Ethanol, benzene, benzamid and DMSO strongly promote the detachment of cortex in the hyalospheres, like in the normal fronts. In the large hyaline ring of the hyalospheres the involvement of cortex in membrane invagination and separation of endosomes, is also clearly seen. They are always separated by cutting the channel at the level of cell surface, with simultaneous disengagement of a cortical sheet. Endocytosis in the hyalospheres is repeated at regular time intervals, exactly at the same spots on the cell surface.

9 *. Motor Polarity of Locomoting Cells of *Amoeba proteus*. Lucyna GRĘBECKA, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The motor polarity of amoeba is not determined at the ultrastructural level by the distribution of contractile proteins: the cortical network

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

10 **. Activity of Surface Membrane-associated Lectin in *Giardia* Strains. W. KASPRZAK and A. C. MAJEWSKA, Department of Biology and Medical Parasitology, Academy of Medicine, 10 Fredry str., 61-701 Poznań, Poland.

F a r t h i n g et al. (1986) investigated lectin-mediated attachment of Portland-1 *G. intestinalis* trophozoites (G) in a model system with erythrocytes (E) and found that G attaches to E *via* a mannose-glucose binding surface lectin. Since mannosyl residues are present on the surface of human intestinal epithelial cells and some dietary lectins are injurious to small intestine, the authors concluded that this lectin may complement the attachment of G to surface epithelium and may contribute to functional and/or morphological damage of small intestine in some individuals with giardiasis.

To confirm this suggestion we examined 23 fresh G isolates from human and 2 isolates from a cat (*G. cati*) and a guinea-pig (*G. caviae*) using a mixed-agglutination microassay (serial doubling dilution of G, initial concn. 1×10^7, mixed with 1% trypsinized rabbit E, incubated in PBS-bovine serum albumin with 0.625 mM Ca, pH 6, in 23°C, for 1 h). The results showed that all G isolates have surface lectin(s) with specifities for saccharide residues on the experimental rabbit E. However, agglutination titer differs for particular G isolates and ranges from 1 : 1

to 1 : 256. The inhibition of agglutination with carbohydrates differs too and indicates no unique pattern of surface membrane — associated modality for adherence to host epithelium.

11 **. Lectin-induced Agglutination of Different *Giardia* Isolates. W. KASPRZAK and A. C. MAJEWSKA, Department of Biology and Medical Parasitology, Academy of Medicine, 10 Fredry str., 61-701 Poznań, Poland.

The surface of parasitic protozoa may be important in adherence on host surface epithelium, in host defense mechanisms and pathogenesis of disease. Hill et al. (1981), using specific lectins (L.), examined surface carbohydrates of Portland-1 *G. intestinalis* strain and found that the parasite most pronouncely binds wheat germ agglutinin (WGA) but appears to expose little surface carbohydrates.

We tested the agglutinability of 20 *G. intestinalis* (G) isolates from human in the presence of WGA, concanavalin A and phytohemagglutinin. Fluorescein isothiocyanate (FITC)-labelled L were also used to determine L binding to G. The microagglutination assay (5×10^5 of G incubated with 400 µg of L in PBS pH 7.2 at 22°C for 15 min) shows that most of the G isolates agglutinate (in groups of $\geqslant 3$) in the presence of ConA with relatively high percentage. Using FITC-labelled L assay (1×10^6 G incubated with 100 µg of FITC-labelled L in PBS pH 7.2 at 4°C for 30 min) we found highest percentage of fluorescent G (up to 23%) with FITC-WGA. Both assay systems did not differentiate between G isolates from symptomatic and asymptomatic cases of giardiasis. The results confirmed the suggestion that G trophozoites expose relatively little surface carbohydrates moieties. Each G isolate appears to have different pattern of surface carbohydrates.

12 *. Fluorescence and Electron Microscope Studies of Membrane-cortex Contacts in the Hyalospheres of *Amoeba proteus*. Wanda KŁOPOCKA, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The EM studies of amoebae preincubated at 35°C, and fixed between 0-15 min after transferring them to room temperature, allowed localization of cortical layer bound to the cell membrane and, in some places, detached from the surface. The detached cortex was present in undifferentiated spheres without separated hyaloplasm as well as in the hyalospheres. Regardless of the distance between the cell surface and the separated old cortex, the new MF layer reconstructed just beneath the plasma membrane was always observed. The structure of cortex adjoining the membrane and that retracted across the hyaline zone, has been identified as composed of a net-work of actin filaments. Thick fi-

laments were not observed within the contractile layers of studied cells. Also the fluorescence methods showed that the contractile cortex can locally lose its contact with the membrane and be detected at different distance from the surface of the active hyalospheres. Labelling of heat pretreated amoebae with FITC-antiactin and Rh-phalloidin confirmed the action structure of the cortical layers detached and retracted within the hyalospheres. It is concluded that both, the contractile activity of the cell cortex and the dynamics of membrane-cortex contacts are responsible for separation of cytoplasm into the hyalo- and granuloplasm, and then for the repeated detaching of this active layer from the cell membrane and its shifting inwards through the hyaloplasmic ring.

13 *. Separation of the Peripheral Hyaloplasm from the Plasma Membrane in Caffeine-droplets of *Physarum polycephalum*. Joanna KOŁODZIEJCZYK and Andrzej GRĘBECKI, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The caffeine-droplets freshly generated from plasmodial veins are rounded forms with reduced motile activity, morphologically resembling the spherical cells of *Amoeba proteus* preincubated in high temperature. Such forms with the cytoplasm separated into hyalo- and granuloplasm (contracted state) manifest spontaneous detaching of optically dense peripheral sheets from the plasma membrane and their shifting toward the granuloplasmic core. This phenomenon is regularly repeated at 6-15 s intervals. The separation of the peripheral hyaloplasm is strongly promoted by some anaesthetics and solvents affecting the membrane properties, especially by 3% ethanol. In the plasmodial caffeine-droplets we also observed the formation of pinocytotic channels induced by KCl, NaCl and ethanol. The channels with dilated basal region are seen mostly in the close proximity of granuloplasm. Formation of the pinocytotic channels can be repeated several times at one and the same place on the droplets surface. Both observed phenomena: the separation and retraction of cortical sheets, and the pinocytosis, demonstrate the high dynamics of the contractile filamentous layer associated to the membrane of plasmodial caffeine droplets.

14 *. The Effect of Pyrethroid Insecticides on *Paramecium primaurelia*. Zofia KOMALA, Department of Systematic and Experimental Zoology, Polish Academy of Sciences, 17 Sławkowska str., 31-016 Kraków, Poland.

Paramecium as a representative of aquatic environment effected by various toxic substances contitutes a very convenient test (K o m a l a Z. 1982: Bull. Environ. Contam. Toxicol., 28, 660) material for the evaluation of the toxicity of different pesticides. Paramecia used for bioassay

test derived from homogenous culture being in a synchronized stage of life. Dilutions of the pyrethroid insecticides Cymbush 25 EC and Ambush 25 EC in different concentrations of active substances (cypermethrin and permethrin) were studied. It was found that the both insecticides did not cause disturbances of the fission rate of the vegetative progeny of surviving paramecia and caused no morphological abnormalities of them, in contrast to other pesticides as Gesagard (Komala Z.: 1975, Folia Biol. (Kraków), 23, 231), and Cartap (Komala Z.: 1982: Bull Environ. Contam. Toxicol., 28, 660) also tested on the paramecia.

15 *. Polyadenylated RNA During DAPI-arrested Regeneration of *Tetrahymena* cilia. W. KRAWCZYŃSKA, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa and B. KŁUDKIEWICZ, Department of Comparative Biochemistry, Institute of Biochemistry and Biophysics, 36 Rakowiecka str., 00-532 Warszawa, Poland.

Deciliation of *Tetrahymena* cells induces increase of α- and β-tubulin mRNA, as well as a synthesis of a new protein molecules, independently of intracellular pools. The recovery of cell motility is a good marker of molecular events connected with cilia regeneration process.

Regeneration of cilia in *Tetrahymena* cells treated with 1 μg/ml of 4,6-diamidine-2-phenylindole. 2HCl (DAPI) takes place about 30 min later then in untreated controls. Analysis of RNA isolated at 50 min of reciliation, that is when the control cells — but not DAPI-treated, have just restored their motility revealed that: 1 — two peaks of polyadenylated RNA were present both in DAPI-treated cells as well as in control ones; 2 — the mobility of these poly (A) rich RNA was the same in two compared cell samples and can be evaluated as 26 S and 18 S; 3 — in cell-free translational system these poly (A^+)-RNA directed the synthesis of α- and β-tubulin; 4 — in DAPI-treated cells, the lower amount of mRNAs was found. Therefore, we suggest that the retardation of cilia regeneration in DAPI-treated cells is caused by diminision of the amount of mRNA for α- and β-tubulin.

16 *. Motility of Cytoplasm during Division in Aposymbiotic *Paramecium bursaria*. Marzena KRUCIŃSKA and Jerzy SIKORA. Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

Cells of *Paramecium tetraurelia* and *P. bursaria* show cytoplasmic streaming during their whole life with the exception of cell division period. Cyclosis has a determined velocity characteristic of each species in definite circumstances. Aposymbiotic *P. bursaria* cells of 20-23 h generation time at 21°C were examined. A new method of immobilization was used. Specimens were isolated into small drops of grass medium

covered with paraffine. Nomarski system was used to examine cells which attached to the microscope slide surface. Observations were made at 25°C. Cytoplasmic streaming stops about 30 min before cells separate when cleavage furrow and new oral apparatus are clearly seen. In sister cells cyclosis starts to move about 35 min after division and gradually reaches interphasal velocity.

17 *. Motor Response of Intact Cells *Fabrea salina* and its Fragments Towards K/Ca Factor in External Medium. A. KUBALSKI, A. ŁOPATOWSKA and S. DRYL, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

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 conclusion 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.

18 *. Influence of Localized Photic Stimuli on the Detachment of Cell Cortex from the Membrane in the Hyalospheres of *Amoeba proteus*. Ewa N. KWIATKOWSKA and Andrzej GRĘBECKI, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The detachment of cell cortex from the membrane, such as it is manifested by the hyalospheres, in normal amoebae is responsible for the formation of new fronts of locomotion. It is generally known that illumination of the tips of advancing pseudopodia provokes their retraction, while shading or elimination of the blue light component activates their extension. Therefore, the reaction of hyalospheres to the same photic stimuli was investigated. The hyalospheres which spontaneously manifested serial separation and inward retraction of successive cortical sheets, were examined. The detachment of cortex was blocked after exposure of the whole hyalosphere to the intense light, and was soon resumed when the whole cell was shaded again. In other experiments the light-shade borderline was so adjusted as to intersect one hyalosphere. Then, the detachment of successive cortical sheets was continued (or initiated) only in the shaded part of the cell, whereas it almost immediately ceased on the bright side. When the positions of the bright and

dark zones were mutually inverted, the hyalosphere quickly adapted to the new asymmetry of the stimulus, i.e., the cortical activity was stopped on one side and restored on another. Moreover, the light-shade difference established across a hyalosphere often resulted in translocation of the hyaloplasm into the shaded area, with a respective shift of the granuloplasmic core toward the illuminated zone and closer to the cell membrane.

19 *. Bacteriolytic Enzymes from *Acanthamoeba castellanii*: Evidences for the N,O-diacetylmuramidase Activity. Lucyna MĄDRA, Danuta DROŻAŃSKA and Wincenty DROŻAŃSKI, Department of General Microbiology, Maria Curie-Skłodowska University, 19 Akademicka str., 20-033 Lublin, Poland.

Two forms of bacteriolytic N,O-diacetylmuramidase isolated from *Acanthamoeba castellanii* were characterized. The maximal yields of enzymes were obtained by thermal desintegration of shaken axenic cultures of *A. castellanii* which were grown for five days at 28°C in proteose peptone-yeast extract-glucose medium. The purified enzymes were shown to be basic proteins of low molecular weight. Both enzymes exhibited maximal activity at the acidic environment (pH 5.0 and pH 5.6 for muramidase "A" and "B" respectively). The activity was maximum in 0.02 M sodium acetate buffer. A higher ionic strength inhibited cell walls hydrolysis. The enzymes lysed peptidoglycans isolated from gram-negative bacteria as well as whole cells and peptidoglycans of gram-positive bacteria even those lysozyme resistant. However, both enzymes were inactive towards peptidoglycan with N-unsubstituted glucosamine residues. Each enzyme were shown to act as muramidase cleaving the glycosidic linkages of the cell wall peptidoglycan between N-acetylmuramic acid and acetylglucosamine and yielding exclusively uncrosslinked disaccharide peptide monomers and the peptide-crosslinked bis disaccharide peptide dimers. The specificity of the enzymes was established towards lysozyme resistant peptidoglycan of *Proteus mirabilis* R45, where N-acetylmuramic acid residues are O-acetylated on C_6, to be N,O-diacetylmuramidase.

20 **. A Study on the Occurrence of *Kudoa alliaria* Schulman et Kovaliova 1979 (*Protozoa: Cnidosporidia*) in *Micromesistius australis* (*Pisces*: *Teleostei*) from the Falkland Fishing Grounds. W. PIASECKI and M. MOŻDŻER, University of Agriculture in Szczecin, 8 Janosika str., 71-424 Szczecin, Poland, Deep Sea Fisheries Company "Gryf".

Studies were carried out during a commercial cruise of M/T Korwin. The data collected have a practical value since *M. australis* is the "number one" fish species in Polish deep sea catches. *Kudoa alliaria* spores

grouped in big cysts were found in the fish muscles. From 7 July until 4 October 1986, 175 hauls were taken yielding a total of 2421 tonnes of *M. australis*. 5250 fish individuals were studied (30 from each haul). Their weight, length and gonad maturity as well as number of *K. alliaria* cysts per 250 g muscle sample (fillet) were determined. An average number of cysts was 7.38. It increased throughout the season from 4.62 to 10.92. There were no *K. alliaria* — free hauls and non infected fishes were very rare. In many hauls over 15 cysts per 250 g muscle (on the average) were found.

21 *. Chromosomes of *Paramecium jenningsi* (*Ciliophora, Protista*). Ewa PRZYBOŚ, Department of Systematic and Experimental Zoology, Polish Academy of Sciences, 17 Sławkowska str., 31-016 Kraków, Poland.

Studies on *P. jenningsi* chromosomes were carried out using the new method (Jurand A. and Przyboś E. 1984: Folia Biol. (Kraków), 32, 295-300, Przyboś E. 1986: Folia Biol. (Kraków), 34, 133-166) of analysis of 0.5 μm serial sections of dividing paramecia fixed and embedded as for the EM but after staining with toluidine blue examined in a light microscope. The total diploid number of chromosomes in a particular micronucleus in anaphase of mitosis was estimated by adding the numbers of chromosomes found in its successive serial sections. Applying that method, far more precise than the previous squash method, the karyological differentiation of *P. jenningsi* strains within one genetic species was found.

22 *. Effect of Starvation of Aposymbiotic *Paramecium bursaria* on the Velocity of Cytoplasmic Streaming. Jerzy SIKORA and Anna WASIK, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The particulate material of the surrounding medium of *P. bursaria* evokes the increase of the food vacuoles formation rate and the cytoplasmic streaming velocity. Stimulating effectiveness is proportional to the concentration of the suspension used. *Paramecium* shows vigorous cytoplasmic streaming throughout its life cycle, beyond the period close to the cell division and the conjugation, however, the velocity might vary without a clear cause. Since the rate of phagocytosis depends on the duration of the preceding starvation, it was examined whether this factor is responsible for the changes of the cytoplasm motility. It was stated that the *P. bursaria* starved in the mineral maintenance solution and then fed with the latex suspension (6×10^8 particles ml^{-1}) during 3 min and afterwards immobilized with $NiCl_2$ solution, shows the decrease in cytoplasmic streaming velocity: 9% after 3 h of starvation, 15.5% after 12 h and 27.5% after 24 h. Therefore it seems reaso-

nable to suppose that the degree of starvation of aposymbiotic *P. bursaria* may affect the velocity of the cytoplasmic streaming.

23 **. The Comparison of Sarcocystosis of Skeletal Muscles in Buffaloes (*Bubalus bubalis*), Bisons (*Bison bonasus*) and in Black-White Lowland Breed Cows. Józef SZAREK and Tadeusz ROTKIEWICZ, Department of Pathological Anatomy, Faculty of Veterinary Medicine, Academy of Agriculture and Technology, Kortowo 105-D, 10-717 Olsztyn, Poland.

In this study 9 bisons living in N.E. forests in Poland, 43 buffaloes slaughtered in Maharashtra state (India) and 43 black-white lowland breed cows were examined post mortem. Macroscopic and microscopic studies of skeletal muscles had been carried out according to the method described in paper: Szarek et al. 1987: Revta iber. Parasit., 47, 229-235.

The greatest skeletal muscles susceptibility on sarcocystosis was stated in buffaloes (39.5% examined animals), lower in cows (16.3%) and the lowest in bisons (11.1%). Whereas the number of sarcocysts present in buffalo muscle sections was almost three times higher than in cows and four times higher than in bisons.

Parasites present in muscles most frequently caused retrogressive changes, rarely inflammations and the most rarely circulatory disturbances and progressive changes in adherent sites. The number of changes was directly proportional to sarcocyst numbers. The clearest histopathological changes were observed in buffaloes, much less clear in cows and the least clear in bisons.

24 **. Phagocytosis of Gonococci by *Trichomonas vaginalis*. Electron Microscopy Studies. Hanna SZRETER and Jerzy KASSNER, Institute of Microbiology, University of Wrocław, 63/77 S. Przybyszewskiego str., 51-148 Wrocław, Poland.

Cells of *T. vaginalis* after centrifugation from the culture were incubated with *Neisseria gonorrhoeae* in saline for 30 min. Non phagocyted bacteria were removed and two samples of *T. vaginalis* containing phagocyted gonococci were incubated for 30 and 270 min respectively. They were processed for electron microscopy by standard glutaraldehyde and osmic acid metod. Intensive phagocytosis of gonococci by *T. vaginalis* has been observed. Gonococci may be ingested as separate cells or (more often) as cell clusters; up to several microorganisms may be seen in one phagosome. Maximal observed number of phagocyted gonococci in one protozoan cell was 60. Inside phagosomes seemingly intact bacteria may be seen even at the prolonged incubation as well as partly disintegrated ones and membranous structures being probably

rests of digested gonococci. Numerous bacteria are found in cytoplasm outside phagosomes showing no structural changes. Bacteria inside phagolysosomes show different electron density which may be due to different degrees of their destruction.

Phagocytosis of *N. gonorrhoeae* by *T. vaginalis* differs markedly from previously described by us phagocytosis of *Streptococcus faecalis*.

25 *. Dichloroisoproterenol does not Inhibit the Motility of Cytoplasm in *Paramecium bursaria* Cells. Anna WASIK and Jerzy SIKORA, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

The particles of the suspension present in the medium, stimulate either the phagocytosis and the cytoplasmic streaming velocity in *Paramecium bursaria*. It was demonstrated that the catecholamine antagonist — dichloroisoproterenol (DCI) evokes the distinct decrease of the phagocytose activity of the cells, however, does not influence the rate of the cytoplasmic flow. The rate of phagocytosis was evaluated as a number of food vacuoles formed during the first 3 min exposure of the cells to the carmine suspension (6×10^8 particles ml^{-1}). When the ciliates were incubated for 3 min in the medium containing 12.5 $\mu g\ ml^{-1}$ DCI the rate of phagocytosis was three times lower than in the control, whereas the cytoplasmic streaming velocity remained almost on the same level as in the DCI nontreated cells. Results lead to the conclusion that the inhibition of the phagocytosis does not cause the changes in the flow of the cytoplasm velocity. Therefore one may postulate that in *Paramecium bursaria* exist two independent paths of the regulation of phagocytosis and intracellular cytoplasmic movement.

26 *. *Paramecium* Phagocytosis is Controlled by β-receptor-coupled Adenylate Cyclase System. Elżbieta WYROBA, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warszawa, Poland.

Studies on inhibition and stimulation of *Paramecium* phagocytosis lead to conclusion that this process is under the control of β-receptor-coupled adenylate cyclase system. β-receptor antagonist 1-propranolol exerts a dose-dependent inhibiting effect on *Paramecium* (stock 299s axenic) phagocytosis and completely blocks it at 70 μM when the cells are pretreated with this drug for 20 min. Forskolin — an unique activator of adenylate cyclase evokes a two-fold increase in phagocytic activity during 10 min of continuous treatment when added simultaneously with latex beads. A stimulation effect of forskolin on phagocytosis is much higher in the case of the ciliates in which such activity had been previously blocked by pretreatment with 1-propranolol.

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Ehret C. F. and Powers E. L. 1959: The cells surface of *Paramecium*. Int. Rev. Cytol., 8, 97-133.

Gelei J. von 1939: Das aussere Stutzgerustsystem des Parameciumkörpers. Arch. Protistenk., 92, 245-272.

Titles of references are given in their original language (not translated). In papers written in English, French or German, the Cyrillic type of the Russian is transliterated according to the international system (ISO Recommendation R 9 September 1954). This regulation is not applied to names if there exists their traditional spelling. Also author may freely choose in transliteration of his own name. In Russian papers, the Russian references are cited in Cyrillic, the others in the Latin characters, but they must be listed all together in the Latin alphabetical order.

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