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Morphological Variability of *Trichodina reticulata* Hirschmann et Partsch, 1955 (*Ciliata, Peritrichida*), a Parasite of *Carassius carassius* (L.) from Small Pond in Kortowo (Olsztyn)

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Synopsis. Morphological variability and its sources have been studied in ciliates, *Trichodina reticulata* Hirschmann et Partsch (*Peritrichida*) parasitizing crucian carp, *Carassius carassius* (L.) from a small pond in Kortowo (Olsztyn). High and statistically significant variation has been noted between groups of trichodinas collected in cool and warm seasons of the year, as well as slightly lower but also significant variation among particular subpopulations. The sources of such type of variation in comparison with variation noted n other species of trichodinas have been discussed.

Interpopulational variation in fish parasitizing trichodinas has been the object of only one publication till now. Kazubski and Pilecka-Rapacz (1981) studied, in populational aspect, morphological variability in *Trichodina nigra*, parasitizing gills and skin of perch-pike (*Lucioperca lucioperca*) from Szczecin Bay. Formerly the interpopulational variation had been studied in trichodinas parasitizing the urinary bladder of newts and frogs (Kazubski 1979, 1980) and the mantle cavity of slugs (Kazubski 1981). The aim of the present paper is to elucidate this phenomenon in *Trichodina reticulata* Hirschmann et Partsch, occurring on the body surface of crucian carp, *Carassius carassius* (L.), living in a small pond in which great seasonal fluctuations of water temperature occurred.

Material and Methods

The trichodinas used in the present paper were collected in 1963–1965 from crucian carps, *Carassius carassius* (L.), from a small pond in Kortowo (Olsztyn). This pond, measuring some thousands of square metres, is situated to the south from the Kortowskie lake and from two sides is surrounded by high banks. During investigation the banks were not overgrown by trees so the temperature of water was high due to strong insolation in summer. The material was collected in

spring, summer and early autumn. Precise dates of particular samples collection are given in Table 1. The fish used for investigation were small, their body length did not overpass several centimetres. They were catched near the banks of the pond.

The trichodinas were collected mainly from the body surface of examined fish. The term "subpopulation" is applied to a group of ciliates from a single host specimen, similarly as in former papers (Kazubski 1979, 1980, 1981, Kazubski and Pilecka-Rapacz 1981).

Measurements of the body and of the adhesive disc were made on silver impregnated preparations after Klein. Most samples of examined subpopulations comprised about 30 ciliates. The measurements were made according to the principles described in previous papers (see above). The following features were measured: (1) body diameter, (2) diameter of the adhesive disc with the border membrane, (3) diameter of the adhesive disc without the border membrane, (4) diameter of the denticulate ring, (5) number of denticles, (6) length of the denticles. Moreover, for each subpopulation mean length of an arch of the denticulate ring corresponding to one denticle was counted according to the formula:

$$\frac{\text{Denticulate ring diameter}}{\text{Mean number of denticles}} \times \pi.$$

The variation in trichodinas was studied with the aid of statistical methods. Mainly the an lysis of variance was made using two level nested ANOVA with unequal sample sizes (Sokal and Rohlf 1969, Box 10.4), with computation of expected mean squares and degrees of freedom. The analysis was made for three features: (1) diameter of the adhesive disc without the border membrane, (2) diameter of the denticulate ring, and (3) the number of the denticles. Choice of these characters, regarded as representative for trichodinas, was argued by Kazubski (1979).

Variation among subpopulations and variation among groups of subpopulations from various seasons of the year have been tested. Three groups of subpopulations have been distinguished: spring group (collected in March), summer group (June to August) and autumn group (September). The analysis of variance has been made in order to verify the hypothesis on the occurrence of seasonal variation in these trichodinas.

Results

The values of metric and meristic characters of examined subpopulations of *Trichodina reticulata* are given in Table 1. The mean values for analysed seasonal groups of trichodinas are also included in this Table. The values concerning particular subpopulations show a moderate differentiation being more clearly pronounced in seasonal groups. The differences between these groups have a defined character. Mean dimensions of the body, of the adhesive disc and of the denticulate ring in trichodinas collected during summer are smaller than in those collected in spring (March) and autumn (September); the mean values in September do not yet reach the maximum characteristic of early spring. The number of denticles changes in another way. From March to summer months fairly sharp decrease in the number of denticles may be observed. This decrease, although considerably milder, lasts up to September. After that, the increase of the number of denticles ought to be presumed. However, the general pattern of the trichodinas during examined seasons of the year po not change noticeably.

Table 1

Mean values (M) and standard deviation (SD) of main characters in samples of 23 examined subpopulations and three seasons groups of *Trichodina reticulata* from *Carassius carassius* (*n* — sample numerosity)

Date	No. of fish	Diameter (μm)												No. of denticles			Length of denticles			Width of denticle (μm)
		body			adhesive disc with border			adhesive disc			denticulate ring									
		M	SD	*n*	M	SD	*n*	M	SD	*n*	M	SD	*n*	M	SD	*n*	M	SD	*n*	
16. 3. 1965	297	84.82	7.92	28	66.58	4.80	30	61.75	4.76	30	37.25	3.63	30	27.97	2.20	30	16.55	0.91	30	4.18
31. 3. 1965	298	87.26	8.35	27	71.02	6.01	30	65.63	6.32	30	38.92	4.16	30	26.97	1.38	30	16.98	1.07	30	4.53
—	299	85.95	8.25	28	70.20	6.74	30	65.02	6.63	30	37.77	5.16	30	28.07	2.48	30	16.18	1.13	30	4.23
—	301	90.22	13.70	16	70.84	9.88	16	65.56	9.59	16	38.12	7.37	16	27.38	2.09	16	16.37	1.42	15	4.37
—	302	85.72	10.01	30	70.15	6.37	30	64.87	6.24	30	38.90	4.30	30	27.17	2.00	30	16.90	1.03	30	4.50
—	303	90.05	11.61	20	71.55	5.45	20	66.67	5.23	20	40.33	4.22	20	26.70	1.38	20	16.17	0.95	20	4.75
—	304	90.78	7.69	30	73.93	4.26	30	68.00	4.05	30	41.72	2.59	30	26.37	2.11	30	17.18	1.04	30	4.97
—	305	93.63	11.47	30	73.88	5.47	30	68.83	5.25	30	41.52	3.80	30	27.43	2.30	30	17.32	0.96	30	4.76
8. 6. 1963	88	86.85	10.42	30	68.60	5.52	30	63.48	5.38	30	37.30	3.73	30	25.23	1.55	30	18.30	1.12	30	4.64
	89	81.48	7.61	30	65.18	4.77	30	60.28	4.66	30	35.53	3.49	30	25.50	1.89	30	17.65	0.94	30	4.38
2. 6. 1964	155	82.87	8.36	19	64.95	6.37	19	59.92	6.22	19	35.37	4.35	19	24.37	1.67	19	18.29	1.15	19	4.56
7. 7. 1964	182	81.03	8.01	30	65.53	5.74	30	60.63	5.56	30	35.65	3.44	30	26.87	1.80	30	17.68	0.78	30	4.17
24. 8. 1964	213	83.07	8.38	30	65.71	4.90	31	60.68	4.92	31	34.35	3.13	31	24.16	1.34	31	17.53	0.87	31	4.47
—	214	80.43	8.36	29	64.58	5.04	30	59.43	4.77	30	33.45	3.26	30	23.67	1.18	30	17.75	0.98	30	4.44
—	215	78.28	10.12	30	63.37	4.84	30	58.33	4.73	30	33.08	3.51	30	25.20	1.63	30	17.58	1.20	30	4.12
—	216	84.82	7.01	30	66.17	4.84	30	61.00	4.65	30	34.98	3.35	30	24.97	1.16	30	17.90	0.81	30	4.40
—	217	82.50	10.25	30	64.93	5.11	30	59.88	4.95	30	34.17	3.34	30	25.97	1.71	30	17.55	0.84	30	4.13
—	219	77.25	7.23	30	65.50	4.91	30	60.50	4.66	30	33.57	3.29	30	26.13	1.48	30	17.72	0.90	30	4.04
—	220	81.40	5.92	24	66.02	3.69	24	61.06	3.68	24	34.62	3.10	24	27.12	2.05	24	18.06	0.72	24	4.01
18. 9. 1963	119	86.42	10.47	30	67.88	5.53	30	62.77	5.49	30	36.45	4.12	30	25.73	1.62	30	18.10	1.07	30	4.45
—	120	83.27	8.92	11	68.59	5.88	11	63.82	5.82	11	37.31	3.69	11	25.09	2.02	11	18.32	0.96	11	4.67
—	121	83.23	8.88	13	63.15	4.62	13	58.36	4.44	13	34.27	2.99	13	24.23	1.64	13	17.58	1.15	13	4.44
20. 9. 1963	143	88.80	10.70	30	70.70	5.47	30	65.72	5.33	30	37.37	4.25	30	24.87	1.36	30	18.15	0.83	30	4.72
March		88.45	10.06	209	71.01	6.39	216	65.77	6.24	216	39.35	4.59	216	27.27	2.09	216	16.76	1.11	215	4.53
June—August		81.79	8.77	312	65.52	5.15	314	60.48	5.01	314	34.71	3.58	314	25.38	1.87	314	17.80	0.97	314	4.30
September		86.36	10.21	84	68.25	5.89	84	63.28	5.79	84	36.55	4.04	84	25.11	1.65	84	18.07	1.00	84	4.57
Summarized data		84.73	9.92	605	67.83	6.23	614	62.72	6.07	614	36.59	4.54	614	26.01	2.14	614	17.47	1.15	613	4.42

The analysis of variance made for 23 subpopulations of *T. reticulata* (Table 2) has shown highly significant differences among particular groups of subpopulations in various seasons of the year. In all cases the computed values of F_0 several times overpassed the critical values at 1 per cent risk of error. Differences among subpopulations were lower, but also statistically significant.

Table 2

Two-level nested ANOVA table for three examined characters of *Trichodina reticulata* from *Carassius carassius*

Source of variation	Degree of freedom	F_0-value			Critical value	
		diameter of adhesive disc	diameter of denticulate ring	number of denticles	$F_{0.01}$	$F_{00.5}$
Among groups from various seasons	2	18.301 s	24.677 s	12.679 s	5.85	3.49
Among particular subpopulations	20	3.385 s	3.704 s	6.705 s	1.90	1.59
Within subpopulations	614—23					

Discussion

Trichodina reticulata is a common parasite occurring on the body surface of crucian carps, recorded by many authors in the Central and West Europe. In the present paper the interpopulational variation in this species has been analysed for the first time. The analysis has shown that the interpopulational as well as the seasonal variation are well pronounced in this species, however, the seasonal variation has greater meaning, being dominant.

The present paper is the second one devoted to the analysis of variation in fish parasites. Till now, the populational variation in *T. nigra* from gills and skin of perch-pike in Szczecin Bay (Kazubski and Pilecka-Rapacz 1981) has been investigated. It ought to be mentioned that in both cases the variation among subpopulations was fairly low. It has appeared that the influence of factors other than genetic ones, is more complicated. In *T. reticulata* great and well pronounced seasonal variation is noted (see above), while in *T. nigra* from Szczecin Bay, examined in the period of June–July, only slight diminution of the body dimensions and of the adhesive disc elements, in comparison with the trichodinas collected in May to June, has been observed, being not significant statistically. Both these facts give the evidence for the influence of outer factors (mainly the temperature) on variability in trichodinas. In a small pond in Kortowo the annual fluctuations of temperature are great and cause well marked seasonal variation in *T. reticulata*, while

the great water resorvoir — the Szczecin Bay, undergoes only slight seasonal fluctuations of temperature and the fish living in it may choose the optimum temperature. Thus, the variation conditioned by this factor in *T. nigra* can not be practically noticeable.

The seasonal variation in ciliates of the family *Urceolariidae* was also the object of two other publications in which, however, the populational variation was not taken into account. Reynoldson (1950) described the seasonal variation in body dimensions of *Urceolaria mitra* from a turbellarian *Polycelis tenuis* collected from small pond in the grounds of the Memorial buildings of the University College of North Wales, Bangor. Kazubski and Migała (1968) recorded seasonal variation in dimensions of the body and of the adhesive disc elements as well as in the number of denticles in *Trichodina nigra*, *T. domerguei* f. *acuta* and *T. mutabilis*, occurring on the skin of carps in ponds of Fish breeding Farm in Żabieniec near Warszawa. In all examined species the decrease in body dimensions and adhesive disc elements, as well as in the number of denticles, was observed in summer and the increase of these parameters in cool seasons of the year. In both cases this phenomenon manifested itself in trichodinas inhabiting hosts living in small and shallow water reservoirs susceptible to great fluctuations of temperature. The investigations presented here, as well as the mentioned papers, ascertain the conclusion on the influence of seasonal changes of temperature on variability in ciliates (Kazubski 1975).

A comparison of results of the present paper with former investigations on variation in trichodinas occurring in the urinary bladder of amphibians (Kazubski 1979, 1980) is also interesting. Populations living on the body surface or on gills of fish differ as regards the character and sources of variation from those inhabiting inner organs of their hosts. It seems that in the first case the genetically conditioned interpopulational variation loses its meaning and the variation conditioned by outer factors becomes more pronounced. Supposedly, it depends on the degree of isolation of particular populations being the result of living on the body surface of hosts or inhabiting inner organs by these ciliates.

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Isolation of Amicronucleates in *Paramecium tetraurelia*

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Synopsis. A simple method of isolating amicronucleate paramecia arising in mass cultures is presented. Newly-formed amicronucleates are markedly reduced in length, slow in motion, and, also reduced in fission rate. Amicronucleates originating in mass cultures of chain-formers in stocks d4-78 and also 51 can be detected, and hence isolated based on the first two criteria. Reduction in fission rate provides an addition screening for the amicronucleate cell lines newly-established. Different methods of obtaining amicronucleates are also reviewed.

In common with all ciliates, paramecium possesses two types of nucleus: macronucleus largely responsible for somatic functions and micronucleus for sexual reproduction. Cell lines lacking the micronucleus, i. e., amicronucleates, are of experimental interest in that they allow a study of other functions of the micronucleus in addition to its germinal role. It is now obvious that the micronucleus plays an important role in cellular growth (for reviews on earlier works on ciliates see Taylor and Farber 1924, Kimball 1941, Beers 1946 and Wells 1961) and morphogenesis (Diller 1965, Mikami 1979, Fujishima and Watanabe 1981, Ng and Mikami 1981 b). In addition, amicronucleates provide a powerful tool for the study of problems of nucleo-cytoplasmic interactions (Levine 1953, Sonneborn 1954, Ossipov and Skoblo 1973, Brygoo et al. 1980), especially in conjunction with the nuclear transplantation technique (Fujishima and Hiwatashi 1978, 1981, Ng 1981, Ng and Mikami 1981 a).

Amicronucleates sometimes arise 'spontaneously' in cultures. They can be obtained also by various surgical and chemical methods (see Discussion). However, the majority of these methods require either technical dexterity, or, elaborate isolations only to give a very low yield. This report draws attention to a simple and efficient method of isolating amicronucleate *Paramecium tetraurelia*, based on certain prior observations on amicronucleates shortly after their origination (Ng and Mikami 1981 b).

Materials, Methods and Results

The success of any method of isolating amicronucleates from a mass culture of micronucleates depends on an *in vivo* detection of the amicronucleates. A study of amicronucleates generated by enucleation with a micropipette (Ng and Mikami 1981 b) indicates that several fissions after enucleation the amicronucleates are as a rule small. Measurements made at about 5 fissions show that the cell length of the amicronucleate progeny is reduced by 15% on the average, and 30% in some cells (mean (range) values are 90(75–103) μm vs 107(93–123) μm for bimicronucleate controls; measurements excluding pre- and post-divisional stages). In addition, the amicronucleates in this stage are generally slow swimmers, their fission rate is also markedly reduced (2–3 fissions/day vs. 4–5 for bimicronucleates). Only later do the amicronucleates increase in size and fission rate. This finding suggests the possibility of isolating amicronucleates first arising in a mass culture of micronucleates by recognition of their distinctively smaller size and sluggishness.

This possibility was tested by employing stock d4-78 of *Paramecium tetraurelia*, homozygous for the gene ch1 (Sonneborn 1975). In depression cultures a substantial portion (usually 5–10%) of the cells characteristically fail to complete constriction in cell fission and form chains of two (sometimes three or four) cells. Our study showed variations in the number (0–3) of micronuclei in frequently no less than 10% of either the anterior or the posterior member of these chains. To isolate amicronucleates, several dozens of chains with broad connections between the proter and opisthe were kept in a depression. On the next day a search was then made among the progeny of the chains for cells that were small and slow in motion. These cells were then isolated and each allowed to proliferate into a cell line in a day or two. Some post-divisional micronucleate cells that were small might have been picked up also, but those cell lines growing slowly and producing uniformly small and sometimes thin cells were likely to be amicronucleates. This was then confirmed by aceto-orcein staining of a sample of their progeny.

This method of isolation of amicronucleates based on their small size, sluggishness and also slow growth soon after their origination has also been applied successfully to stock 51, the standard strain, of *P. tetraurelia*. Stock 51 does not normally form chains in cerophyl medium bacterized with *Enterobacter aerogenes*. But sometimes chains appear spontaneously in the culture, probably as a result of bacterial contamination (DeLamater 1939, Keim and Hanson 1964). On several occassions a large number of chains with variations in the number of micronucleus arose and, when collected yielded amicronucleate cell lines as above.

Amicronucleate cell lines can best be maintained in mass tube cultures within the first 15 fissions after their initiation. This is because within an amicronucleate cell line many cells in this period do not survive as a result of gross abnormality in the oral apparatus (Ng and Mikami 1981 b). There after daily isolation lines can be easily maintained as oral morphogenesis becomes more normal (Ng and Mikami 1981 b).

Discussion

Early workers starting at the turn of the century obtained amicronucleate ciliates by regeneration following merotomy (see reviews by Taylor and Farber 1924, Balamuth 1940). The technique of withdrawing the micronucleus with a micropipette, if acquired, is the quickest and cleanest way of generating amicronucleates (in *Euplotes patella*, Taylor and Farber 1924, in *P. caudatum*, Fujishima and Hiwatashi 1978, Mikami 1979, Fujishima and Watanabe 1981, in *P. tetr-*

aurrelia, Ng and Mikami 1981b). The cells appear to be able to recover quickly from the physical injury done by the micropipette.

The micronucleus also can be induced to disappear by ultraviolet irradiation (in *P. putrinum* Fokin 1978, 1979, in *E. aediculatus*, Kloetzel 1980, in *P. tetraurelia*, Sonneborn et al. 1953 — see Brygoo et al. 1980, in *Stylonychia mytilus*, Ammermann 1970) by X-irradiation (in *Tetrahymena pyriformis*, Wells 1961, in *S. mytilus*, Ammermann 1970) and by laser microbeam irradiation (in *P. tetraurelia*, Ng 1980). The general damage of the cell may be minimized in cases when the irradiation is focused on the micronucleus (Ammermann 1970, Fokin 1978, 1979, Kloetzel 1980, Ng 1980), even so, the possibility exists that submicroscopic pieces of the irradiated micronucleus may remain in the cell. The laser microbeam method does sometimes generate minimicronuclei (about 1/3 normal diameter) and also 'ghosts' without stainable DNA material inside the nuclear envelope (Ng, unpublished). Cells bearing such abnormal micronuclei are essentially functional amicronucleates, because no normal macronuclear anlagen could be derived from such micronuclei during autogamy. Such functional amicronucleates can be employed in genetic studies in much the same way as 'true' amicronucleates; in a sense they are better material because they are more vigorous than the 'true' amicronucleates (Ng, unpublished). In addition, they themselves are interesting objects of study. As an alterative to micropipetting, irradiating the micronucleus is efficient in generating amicronucleates.

Both micropipetting and irradiation require a somewhat elaborate set-up and skill, but the method of chemical treatment of the cells does not. Ciliates could be induced to lose their micronuclei by colchicine (treatment of conjugants, in *P. primaurelia* and *P. tetraurelia*, Butzel 1953, in *P. tetraurelia*, and *P. caudatum*, Mikami 1979), urea (in *P. caudatum*, Miyake 1955, 1956), acriflavin (in *P. caudatum*, mentioned in Miyake 1956), hydroxyurea (treatment of conjugants, in *E. aediculatus*, Kloetzel 1980), and also vinblastine sulfate (in *P. tetraurelia*, Brygoo et al. 1980). The details in Miyake's reports indicated that the amicronucleates originated as a result of unequal distribution of the micronuclei in chains or monsters resulting from urea treatment. But some evidence suggested that vinblastine sulfate caused more direct and drastic damage on the micronucleus, sometimes resulting in minimicronuclei (Sonneborn, personal communication). To be effective, sublethal doses of colchicine and vinblastine sulfate have to be applied, so that up to 60% cell mortality might be encountered. Such amicronucleates obtained were frequently not as vigorous compared to those obtained by micropipetting (Sonneborn, personal communication, Ng and Mikami 1981 b) or according to the method in the present report. It is clear that the cells suffered from undesirable side effects of the drugs. How efficient these methods are depends in part on how readily amicronucleates can be detected, once generated in mass cultures. The criteria for detecting amicronucleates (small size, slow motion and also slow growth) emphasized by the present report may have a general application, provided that such are prominent features of amicronucleates in at least some stages,

especially soon after their origination. In this connection, amicronucleate *P. caudatum* became shortened by 15–20% only 6 days (11 fissions) after their origination (Miyake 1956, Fig. 5): thus newly-formed amicronucleates of this species may not be that readily detected based on size difference in mass cultures.

Amicronucleates also arose spontaneously in cultures as a result of degeneration of the micronucleus, forming 'ghosts' (in *P. bursaria*, Woodruff 1931, Chen 1940), irregularities in conjugation (in *P. caudatum*, Diller 1940, in *Didinium nasutum*, Patten 1921) and, unequal distribution of micronuclei to daughter cells during fission (in *P. caudatum*, Diller 1940, Wichterman 1954). To be able to isolate amicronucleates following these phenomena, the amicronucleates would have to be generated with high frequencies, or, morphologically distinguishable from micronucleates. The present work on isolating amicronucleates from chains arising spontaneously in stock 51 cultures illustrates how this could be facilitated based on size difference.

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The Ethogram of *Oxytricha bifaria* Stokes (*Ciliata, Hypotrichida*). II. The Mating Behaviour

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Synopsis. The behaviour of sexually competent cells of *Oxytricha bifaria* Stokes has been studied by means of a video tape recorder, in an attempt of recognizing and describing its elementary patterns.

After mixing complementary types, the "waiting period" ensues, during which the cells slow down ($\Delta_t \simeq 40$ min) and gather into a more or less irregular area ($\Delta_t \simeq 80$–100 min), where the cell-to-cell contacts ("Visible Reaction" = VR) occurs, leading to the eventual pair formation (100–120 min).

If split during the early phases of the VR, the two potential mates perform the "Exploring Reaction" (ER), by which they may either meet again and pair, or leave the areas explored and undergo new "contacts".

The ER consists of a series of jerks, each consisting, in turn, of a backward motion (~ 100 μm), a clockwise rotation ($\sim 65°$), a forward motion (~ 100 μm) and a sudden stop. By the ER the partners scan many times two subcircular areas, partially overlapped. Each jerk is quite similar to the behavioural pattern, called Side Stepping Reaction (Ricci 1981 b), by which a vegetative animal can shift onto new trajectories or even avoid possible obstacles.

Experimental splits of interacting partners after 2, 5, 8 and 11 min from the onset of the VR showed that, being constant the length of the backward and forward motion, the amplitude of the clockwise rotation of each jerk of the ER is respectively of 65, 57, 40 and 34 degrees: the longer are the interactions, the finer is the area-scanning.

Such a differentiation never occurs when the interacting partners belong to the same mating type. Once acquired this kind of cellular "differentiation" is somehow "remembered" for about 20–30 s: after exploring two or three complete round angles, by jerks with angles as wide as about 34°, the partners start making wider and wider jerks, up to the base-value of 65°.

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Introduction

The studies of the preconjugant cell interactions of *Oxytricha bifaria* Stokes (Ricci et al. 1975 a, b, Esposito et al. 1976, Ricci et al. 1980) showed that the mating behaviour constitutes a parameter capable of revealing the fine proceeding of the differentiation(s) occurring during the processes leading to conjugation.

It has to be noticed that the available data on the mating behaviour of various species of ciliates always are given in a few lines of papers more generally devolved to study different problems of their biology, such as the breeding system, for instance: this is the case for *Euplotes patella* (Kimball 1939), *E. eurystomus* (Katashima 1959), *Stylonychia putrina* (Downs 1959), *E. crassus* (Heckmann 1964), *Urostyla hologama* (Heckmann 1965), *E. cristatus* (Wichterman 1967), *Stentor coeruleus* (Webb and Francis 1969), *Pseudourostyla levis* (Takahashi 1973).

Heckmann and Siegel (1964) paid a great attention to the mating behaviour not only describing it particularly in *E. crassus* (cf. also Verni et al. 1978), but also using it as a parameter by which one can distinguish the successive stages of conjugation ("The onset of the mating behaviour ... marks the end of the waiting period"). More recently Cronkite (1979) states that "the end of immaturity comes when mating behaviour is seen", thus showing the relevance of this aspect of the biology of a species, also to judge the general proceeding of the life cycle.

As to the mating behaviour among the species belonging to the genus *Oxytricha*, some data, although very approximate and inaccurate, are already given by Gregory (1923) for *O. fallax* and by Kay (1946) for *O. bifaria*. However, the complex of informations resulting from their papers is almost useless, mainly due to their inexactness. Grell (1951) in *O. fallax* and Siegel (1956) in *O. bifaria* proved to be far more interested in this aspect of the biology and Hammersmith (1976) in *O. fallax* dedicated specifically a whole paragraph of his paper to the "mating behaviour".

On the basis of the results obtained by these authors and by ourselves, a study was planned specifically to study the mating behaviour of *O. bifaria*.

To gain more insight into this field and to get acquainted with the technical problems related to the behaviour of this species an accurate study of its motor behaviour had been planned and carried on previously (Ricci 1981 b).

Materials and Methods

O. bifaria was cultured according to the technique recently standardized by Ricci et al. (1980 a), and kept at 22°C ± 1°C. Cells belonging to complementary types were then mixed and put between slide and coverslip in a moist chamber. After a waiting period of about 80–100 min, the Visible Reaction (VR) began and regularly led to the pair formation, thus indicating that the exam-

ination technique was enough appropriate being the time of pair formation identical to that obtained in cells mixed in depression slides.

The goal of this series of experiments was to study one by one as many VR as possible: for such a reason, no effort was made to have synchronous pairing reaction, being interested into a continuous, even if quantitatively poor, pairing, rather than in a massive reaction. The slides were scored by a dissecting-stereo-microscope until the first VRs became visible and, thereafter, carefully studied by a Leitz-Orthoplan Microscope, coupled with a T.V. camera Philips LDH 4310/00 and with a video tape recorded Philips Fi 340 A/00.

To split the interacting cells, glass needles as thin as 30–40 μm were used. As to this topic, we have to point out that the best results have been obtained with relatively thick needles (about 100 μm), with short, cone-shaped tip: this sort of needle, infect, lends itself to be handled easily and finely, so that an accurate manipulation of VR is possible. The needle was introduced between the slide and the coverslip, to disturb two interacting partners and to evoke their Exploring Reaction (ER). The video recordings were then scored frame by frame and the movements of animals recorded on transparent triacetate sheets, put on the T. V. monitor: in such a way they were made suitable of quantitative analysis (cf. also Ricci 1981 b).

Results

(1) The Behaviour of *O. bifaria* During the Waiting Period

Once the complementary types are brought together, a time lag ensues, during which no visible process occurs. During this period, known as the "waiting period", the complementary cells interact with each other by means of mating type specific soluble factors, gamones (Esposito et. al. 1976). From a more specific behavioural point of view, a clear and progressive reduction of the mean velocity of the creeping oxytrichas can be easily recognized (Fig. 1 B). Although the trend of such a reduction

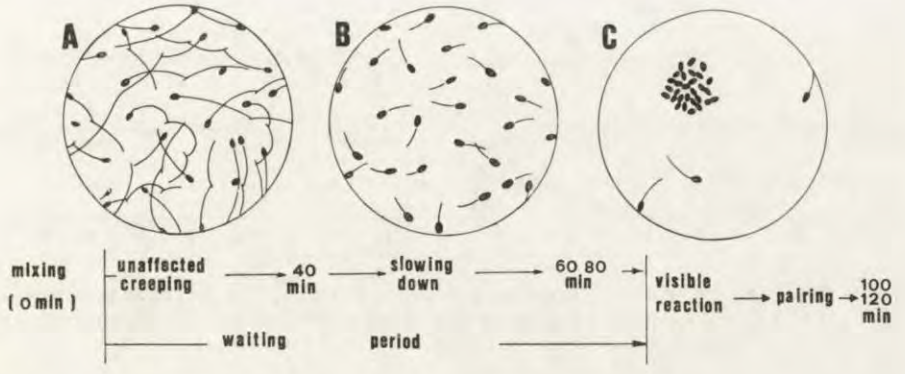

Fig. 1. The behaviour of *Oxytricha bifaria*, during the waiting period. The black lines represent the tracks of the cells: the length is approximately proportional to the creeping velocity. Terminology and times are indicated below the drawings they refer to

in time is difficult to be exactly defined, it can be said that within 50% of the waiting period most of the animals slow down so that their mean speed does not exceed 50 μm/s, namely about 1/10, or even less, of the usual creeping velocity (cf. Ricci 1981 b).

The second behavioural trait, characterizing the mixed cultures, is the progressive clustering of most of the cells within a small, irregularly subcircular area, which can be formed by the animals in any point of the bottom of the depression (Fig. 1 C). Probably due to the reduction of speed, the oxytrichas now bumping against each other do not perform the Side-Stepping Reaction (Ricci 1981 b): therefore, not avoiding each other as they usually do, they make possible the onset of the Visible Reaction (VR).

When about 80–90% of the cells in the experiments are "trapped" in the cluster, the first VRs begin, leading to the pair formation. Almost all of the pairs are formed by cells actually lying within such a cluster.

(2) The Behaviour of *O. bifaria* During the Visible Reaction

Some introductory results of studies of this aspect of the *O. bifaria's* behaviour have been briefly reported by Ricci et al. (1980 a) and by Ricci (1981 a).

The VR (Fig. 2), one of the most stereotyped behaviours of this species, begins

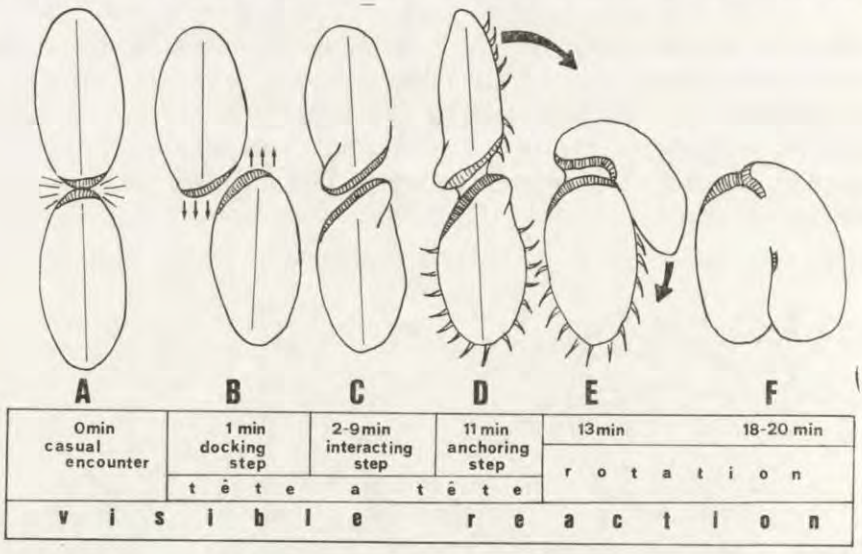

Fig. 2. The behaviour of *O. bifaria* during the Visible Reaction. The cirri have been represented only in D and E, when they play a more specific role. Termilonogy and times are indicated below the stages they refer to. Partially redrawn from Ricci 1981 a

with the casual encounter of the potential partners and it ends when a pair forms, about 18–20 min later. The "*tête-à-tête*" can be described as the first part of the VR, during which the potential mates fruitfully and specifically contact each other.

Three major steps can be recognized and described during this phase: the "docking step" (Fig. 2 B), the "interacting step" (Fig. 2 C) and the "anchoring step" (Fig. 2 D).

After encountering each other, two slowly-and-randomly creeping oxytrichas approach to each other, slowly sliding along their anterior, left edges: this way of behaving perfectly accounts for the term "docking step". After about 1–2 min the partners, lying quite still on the substrate, lift up their anterior ends, so that their peristomial areas and ciliary organelles can interact for as long as about 6–7 min, namely during the "interacting step". After about 11 min from the onset of the VR, the *tête-à-tête* ends up with the "anchoring step", during which one partner anchors the substrate by its marginal cirri, while the other rotates sideways, thus showing to that there is already a pellicular continuity between the mates.

From this moment on, the "rotation" occurs (Fig. 2 E, F), during which one cell lies quite motionless on the substrate, while its partner rotates clockwise around its anterior end, progressively reducing the angle between them from 180°, down to 90° and eventually to about 30–20°. This behavioural pattern does not last more than 4–5 min. Now the pair just formed will resume moving as perfectly and easily as the single cells do (Ricci 1981 b).

(3) The Behaviour of *O. bifaria* During the Exploring Reaction (ER)

The ER is the behavioural pattern shown by the partners, when they are split during the interactions taking place in the VR — very often, it leads them to meet again and to pair. This behaviour can be elicited by natural stimuli (collisions with other cells) and by experimental (mechanical and/or chemical) stimuli. As shown in Fig. 3, the ER consists of a series of many stereotyped jerks, following each other up to scan many round angles. A jerk, in turn, comprehends four successive steps — backward motion (about 100 μm), clockwise rotation (about 65°), forward motion (about 100 μm) and the final sudden stop. The quantitative parameters characterizing a jerk, represented in the lower, left panel of Fig. 3, are quite similar to those of the Side-Stepping Reaction (SSR), the behavioural response by which vegetative and paired animals shift onto new pathways or even avoid possible obstacles (Ricci 1981 b). The repetition of the basic reaction, namely the jerk, seems to be the major difference between the ER and the SSR, and very likely, it is under an endogenous control. Whatever the nature of the trigger for the repetition of the jerks, the partners scan two circular areas partially overlapping in a zone, where they can contact each other again.

If the interacting partners are split later and later in the VR, behavioural responses fundamentally similar to the ER are elicited. However, as shown by circles in Fig. 4, the longer the heterologous cell contacts already occurred, the narrower the angles of the jerks: partners split after 2, 5, 8 and 11 min from the onset of the VR performed ERs, with jerks as wide as 65°, 57°, 40° and 34° respectively. (1) For each value 15 couples of interacting potential partners were experimentally split:

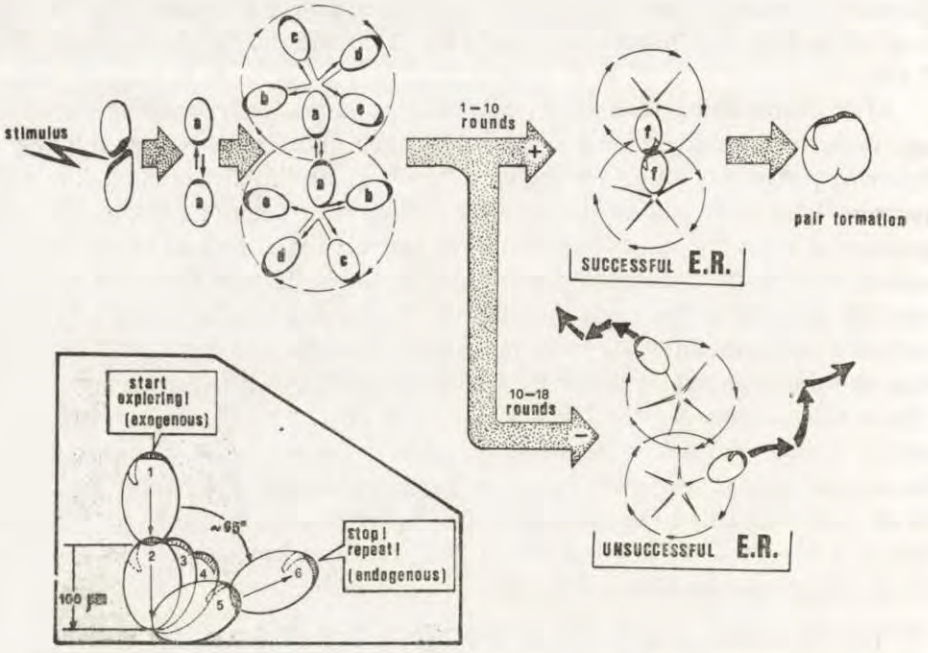

Fig. 3. The Exploring Reaction. The successive steps leading the split partners to meet again and to pair are shown in the upper part (successful ER), while in the lower, right part, an unsuccessful ER has been represented. The lower, left panel indicates the elements of a single jerk of the ER — 1–2 — backward motion, 2–5 — clockwise rotation, 5–6 — forward motion, 6 — stop

their reactions were recorded and then scored at low speed to measure the average angles of jerks. In order to ascertain whether such a progressive differentiation depends upon complementary contacts or not, homotypic *tête-à-têtes* were similarly stimulated and studied. No change in any parameter of the ER was detected, as shown by triangles in Fig. 4, and it was thus concluded that the reduction of the width of the first jerks actually depends upon cell contacts and interactions between complementary, sexually competent systems. The correlation "time-already spent-in-heterologous-contacts" — "reduction-of-angle" might be explained as an adaptive acquisition, enhancing the probabilities for the split partners of meeting again by means of a finer resolution of scanning of their two areas. According to this point of view, the ER could be essentially a behavioural device helping the "lost" partners to meet again or, more in general, helping the species to pass through the bottleneck of sexual reproduction.

Can the partners somehow "remember" their own story? And how long can they do that? (2) The behaviour of 14 couples of cells, split during the late interacting step, was recorded and the angles of all the jerks were carefully measured for the first seven round angles. It was demonstrated that partners split after 11 min of direct interactions, namely at the end of the VR, start scanning the substrate

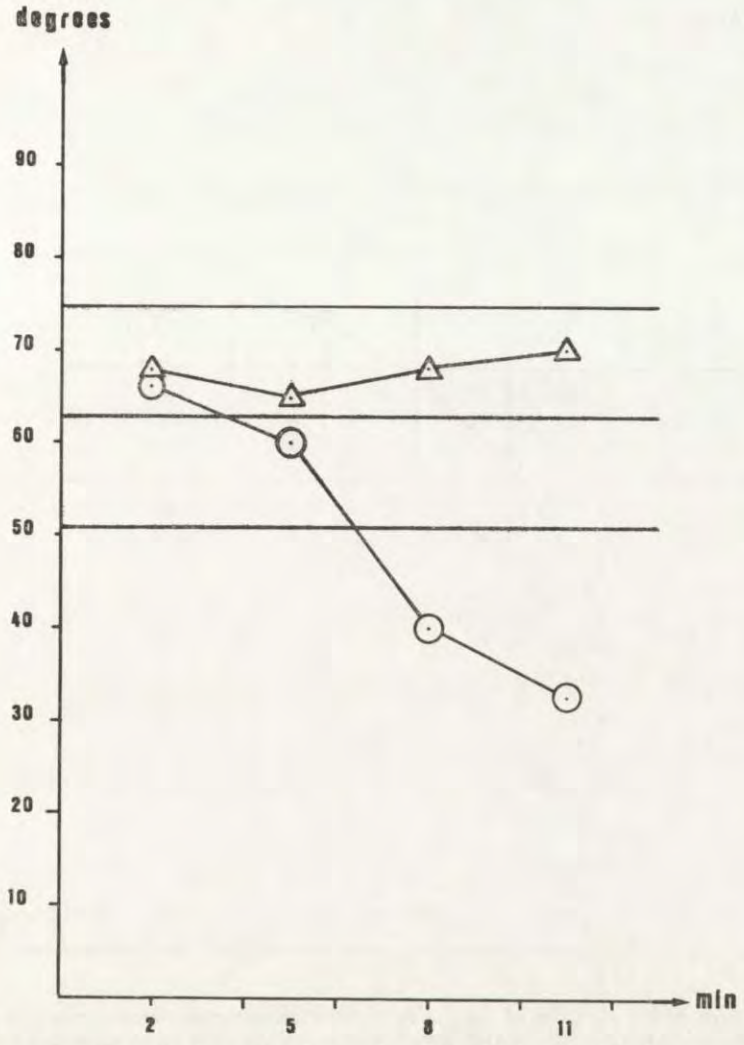

Fig. 4. The progressive reduction of the first angles of the ER (ordinate) is shown as a function of the time lag elapsed from the onset of the VR (abscissa). The black line at about 63° and the shadowed area around it represent the mean value and the standard deviation, respectively, of the angle of the Side-Stepping Reaction (Ricci 1981 b). Circles – heterologous *tête-à-têtes*, triangles – homologous *tête-à-têtes*. Partially redrawn from Ricci 1981 a

by angles as wide as 32–36° for about 2–3 complete round angles (about 20 s) and that after 4 round angles more, the width of the angles progressively rises up to final value of about 65° (Fig. 5). Whatever the molecular basis of the modifications depending on the complementary cell-to-cell contacts, we can conclude that the cells remember them for about 30–40 s, after the splitting stimulus. As the time passes and the ER goes on, however, the general pattern of jerking slowly changes: while, at first, the cells maintain absolutely constant the centre of their rotation,

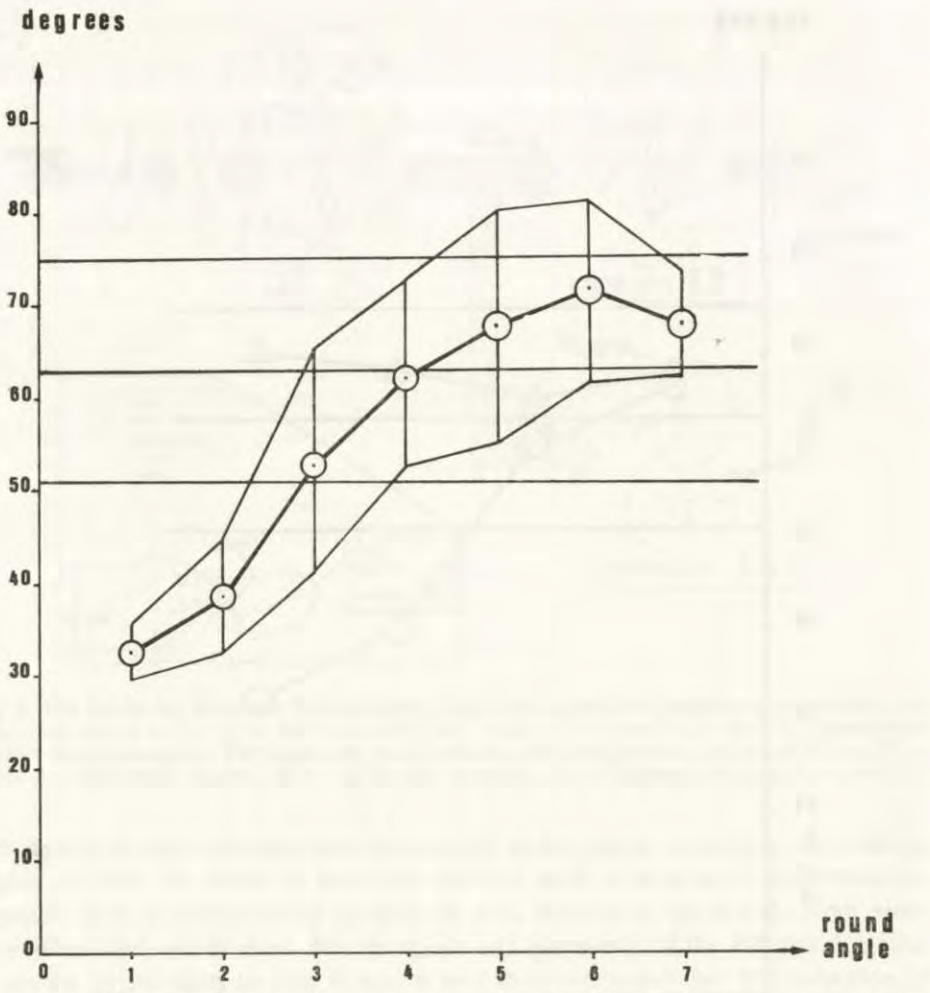

Fig. 5. The progressive widening of the angles of jerks (ordinate) as the partners scan successive round angles during the ER (abscissa) is shown. For each datum the mean value and the standard deviation are indicated. The mean value and the standard deviation of the width of the angle of the SSR are given as in Fig. 4. Partially redrawn from Ricci 1981 a

as shown in Fig. 3, after 10–15 round angles, they start moving it forward of a short space, about 20–40 μm. These two kinds of reactions perfectly mirror the "Radial Reaction" and the "Tangential Reaction", respectively, already known for vegetative cells of *O. bifaria* (Ricci 1981 b). The progressive motion of the centre of the rotation, together with the increase of the angle of the jerks, results into a dramatic dropping of the partners' probability of meeting each other again. When a normal but unsuccessful ER occurs, after 18–20 round angles, the partners start rotating, as the pointers of a clock, around their posterior end, without jerking any more

and just sliding by their ventral surfaces on the substrate. After exploring 2–4 complete round angles in such a way, the ex-partners start creeping forward very slowly and discontinuously, ready to undergo new contacts. The same reaction occurs when a homotypic *tête à tête* comes to its end the animals leave the explored areas

Discussion

The general traits of behaviour leading to the *O. bifaria's* conjugation have been studied in an attempt of drawing a comprehensive and exhaustive picture of this aspect of its reproductive biology. Although already fragmentarily described in previous papers, only recently it was perceived that the behaviour constitutes an essential key to understand the whole complex of what is known about this ciliate.

The data available on the mating behaviour of ciliates seem to mirror a general lack of specific interest in this field: on the other hand, the fact that so many authors (cf. Introduction) spent some sentences or some paragraphs of their papers for the behavioural aspects of the mating processes shows that they had already felt the importance of behaviour in guiding the potential partners to the pairing.

The behaviour of complementary, interacting cells of *O. bifaria* during the waiting period seems to suggest that, as the gamones (Esposito et al. 1976) start mediating exchanges of information between them, a progressive and increasing "inertia" affects the animals, so that their most typical behavioural trait, namely their motility (Sollberger 1962), dramatically drops and almost disappears. The apparent meaning of such a reduction of the creeping velocity is to allow the contacts, which during the following VR, will constitute the prerequisite for the conjugation to occur (Ricci et al. 1980 a). To facilitate these contacts, moreover, a second strategic device was found out by the species: the clustering of the potential partners in a unique "mating arena" (Fig. 1 C). Thus, at a first glance, the general trend of the pre-mating behaviour of *O. bifaria* perfectly fits into what had been described for *O. fallax* (Gregory 1932, Hammersmith 1976) and for *O. bifaria* itself (Kay 1946, Siegel 1956). However, when one tries to compare point by point our data with those previously reported, it becomes evident that there are many discrepancies and several differences, possibly due to the incomplete and fragmentary nature of those papers.

Siegel (1956), one of the most precise authors, describing the mating behaviour of *O. bifaria*, says that "the cells describe contiguous circles" up to contact a partner and that "if conjugation proceeds each cell is supported on the bottom of the dish by its posterior cirri". Neither the "circles", nor "the supporting posterior cirri" have been observed in the mating behaviour of our stocks of *O. bifaria*. In our

opinion, rather than to clonal variations, the differences between the two descriptions are likely to be due to a different attention specifically paid to the behaviour *per se* and, consequently, to a different degree of exactness in the description of the patterns. To conclude, it can be said that, although many descriptions have been already given by many authors about the mating behaviour of many different species of ciliates, hard task will be the comparison, provided that it could be possible, between them and any data newly obtained by specific investigations.

As to the Exploring Reaction, it seems to constitute a sort of behavioural device devolved "to rescue" the partners, split after a certain period of interactions, which would be "lost" for the sexual event of the species. Moreover, this reaction, so important for the *O. bifaria's* reproductive strategy, is as useful as simple, being considerable just as a series of Side-Stepping Reactions. The ER is elicited by mechanical and by chemical stimuli as well: this fact seems to suggest that the ER depends upon internal physiological conditions of the partners, rather than upon the nature of the stimulus itself. These specific, although not yet known, internal conditions would trigger the repetition of the SSR, to perform the ER. It is now interesting to notice that the species exploits a certain, well established and stereotyped pattern (the SSR) to achieve a completely new goal, namely the new encounter of the split partners, without encoding in the genetic memory of the species any new complex behavioural pattern. Such an advantage, however, somewhat costs to the species, which pays it in terms of a lower efficiency of the process itself: the split partners, in fact, will contact each other again by their anterior, right edges, while it would be far more convenient to meet again by the left edges, as it could be possible just rotating counterclockwise in the ER.

Finally, with regard to the mechanisms possibly lying at the basis of the repetition of the SSR pattern to perform an ER, in analogy with what is well known in ciliates such as *Paramecium* (Naitoh and Eckert 1969) and *Stylonychia* (Machemer 1970 b, Machemer and Eckert 1973), it may be put forward the hypothesis of a spontaneous and periodic depolarization of the membrane potential, which would account for the periodic backward creeping of the oxytrichas. Whatever the origin of such a depolarization, it can be supposed that a refractory period follows each depolarization and that during it, the animal creeps forward again for a while: this hypothesis could also explain the progressive passage from the Radial Reaction to the Tangential Reaction, as the ER goes on: the longer and longer forward motions would depend upon longer and longer refractory periods, in turn due to some kind of fatiguing of the cells.

This round of experiments on the mating behaviour of *O. bifaria* seems to indicate that the mating behaviour itself has been too long disregarded, while it deserves the most careful and exhaustive studies, representing one of the most important biological factors, guiding and conditioning the passage of the species through the sexual reproduction, a dramatic process and a severe bottleneck of the lifecycle of a ciliate (Nanney 1977).

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Ultrastructural Changes in the Nuclear Apparatus of *Paramecium bursaria* after Local UV Irradiation of the Generative Nucleus

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Synopsis. The present paper deals with the results of investigation on ultrastructural changes after UV irradiation of the micronucleus in *Paramecium bursaria* and on the dynamics of appearance of fragments of the macronucleus and changes in its ultrastructure resulting of Mi irradiation. It has been found that the irradiation (306 J/m^2) does not lead to the loss of generative nuclei in post-irradiation offspring. However, the Mi in the offspring undergoes the following changes: the longer time from the moment of irradiation the more marked changes appear in the structure of the chromatin and in the configuration of the nuclei. These changes are accompanied by the decrease in DNA content. Selective irradiation of the Mi involves fragmentation of the Ma. The process of invagination of the nuclear envelope and subsequent lobe formation in the Ma has been observed as early as in first hours after Mi irradiation. The process lasts up to 30 days after irradiation, being stopped later. These data show the significance of the Mi during the vegetative life of the cell and the existence of interaction in the heteromorphic nuclear apparatus of ciliates.

The nuclear apparatus of higher ciliates represents a system of heteromorphic nuclei: the macro- (Ma) and the micronuclei (Mi), Morpho-functional features of each type of the nuclei are conditioned probably by various activity of their genomes and by specific internuclear relationships. Possibly, these interactions play an important role in upholding of a defined level and type of the functional activity of the nuclei in ciliates (Ossipov 1978, Fokin 1979 a, Fokin and Ossipov 1981).

Perspective approach to elucidation of the internuclear relationships in ciliates gives the method of experimental impairement or expulsion of one of the nuclei and subsequent examination of changes in morphology and functional activity of the other one (Fokin 1979 a, Kloetzel 1979, Mikami 1979). In our former investigations UV rays were used for the Mi impairment (Fokin and Ossipov 1975, 1981, Borchsenius and Ossipov 1978, Ossipov 1978, Fokin and Borchsenius 1978, Fokin 1978 b, 1979 a). Up to now such studies have been made only at the light microscope level bearing thus on evaluation of the experiments results.

Changes in the Mi morphology due to UV irradiation may be so great that sometimes it is difficult to reveal the nucleus only with the aid of usual cytological methods. To resolve the question of real amicronucleality (Mi^-) of the experimental material, of ultrastructural manifestations of internuclear relationships, and of the nature of pseudomicronuclei found by some authors (Schwartz 1958, Ammermann 1970, Golikova 1978, Fokin 1979 a) the electron microscope studies are indispensable.

In our study on the nuclear apparatus in the offspring of UV irradiated *Paramecium bursaria* an attempt has been made to recognize at the ultrastructural level the dynamics of changes in the chromatin content of the Mi up to disappearance of the generative nucleus, and to evaluate, from the point of view of existing internuclear relationships, temporal morphological reconstruction of the Ma resulting from Mi irradiation (Fokin 1979 a).

Material and Methods

The clones of *Paramecium bursaria* used in this study were taken out from natural reservoirs: clone BP80 from the Borisovka village, Belgorodskij district, in summer 1979, clone T2 from Staryj Petergof, Leningradskij distr., in 1976. Irradiation of the Mi was performed with a device produced by Carl Zeiss, Jena on the base of NF-microscope. Expositional dose — 306 J/m^2. The methods connected with UV irradiation and subsequent cultivation of ciliates were the same as described earlier (Fokin and Ossipov 1975, Fokin 1978 b).

For electron microscope studies the paramecia of BP80 clone with inactivated Mi were fixed within 0.5–7 h after irradiation (series 1). Postirradiation offspring of the cells of this clone was examined during 2 (series 2), 6 (series 3) and 30 days (series 4) after irradiation. Not irradiated ciliates of the clone BP80 were used as control. The offspring of irradiated cells of the clone T2, namely the subclone T2UV-72-2, was examined 3 years after exposition (series 5).

The paramecia were fixed in 2% OsO_4 in 0.2 M cacodylate buffer (pH 7.4) and embedded in araldite. Ultrathin sections were made on LKB-III ultramicrotome with glass blades. The sections were contrasted with saturated uranyl acetate in 70° alcohol and with lead citrate. The material was examined in Hitachi HU-11 e (75 kW) and Tesla BS-500 (60 kW) microscopes.

Results

Control. Ultrastructural organization of the generative nuclei of *P. bursaria* (Schwartz 1976) clone BP80 is typical for this species. Uninterrupted, bimembranaceous envelope with pores is 20.7 nm thick. Its contour is slightly tortuous (Pl. I 1–3). Numerous bunches of microfilaments (Pl. I 2) spread among chromatin aggregations. Chromatin strands (32 nm in the diameter) are evenly distributed in the Mi. Frequently, chromatin elements are situated near the inner membrane of the nuclear envelope (Pl. I 2, 3).

The ultrastructure of the Ma of *P. bursaria* clone BP80 is also typical of the species (Schwartz 1978). Nuclear envelope with pores is composed of two elementary membranes separated by electron clear space (thickness of NE — 20.6 nm) and forms characteristic small invaginations (Pl. I 3, 5). Numerous compact electron dense bodies, 88 nm in the diameter, dispersed in caryoplasma represent the chromatin of the Ma in form of densely packed fibrilles (Pl. I 4). The caryoplasma of the vegetative nucleus comprises not numerous microfilaments (Pl. I 4) and a net of curved threads, being not electron dense. Typical nucleoli are seen in the electron micrographs (Pl. I 5).

Electron clear zone of the cytoplasm adhering to Mi and Ma comprises numerous thin, curled fibrilles and a small number of cisterns of the endoplasmic reticulum (Pl. I 2, 5).

Series 1. The structure of the Mi, irradiated 0.5–7 h before fixation, corresponds with that of the control cells (Pl. II 6, 8, 9, III 13): the inner and the outer nuclear membranes are separated by perinuclear space of equal width (thickness of NE — 21 nm). In spaces among condensed chromatin threads (31 nm in the diameter) bunches of microfilaments are seen; the interphase Mi contains elements of the caryokinetic spindle in form of funnel-like structures of low electron density — the microlamellae, sublamellae of which are joined by microseptae with periodicity 14.5 nm (Pl. II 6).

Basically, the structure of Ma is analogical to that in the control cells: contiguous envelope, composed of two elementary membranes (thickness of NE —20.7 nm), surrounds densely packed chromatin threads measuring 100 nm in the diameter (Pl. III 10, 13). However, the contour of the Ma in a number of cases undergoes some changes — fairly deep invaginations are formed and the lobes begin to develop (Pl. III 11, 12).

Perinuclear zone of the cytoplasm contains numerous canals and cisterns of the endoplasmic reticulum (Pl. II 6, III 10). Irradiation of the Mi does not cause any damage in the structure of mitochondria — in all variants of theex periments the structure of mitochondrial membrane was the same as in the control ones (Pl. I 2, II 7, V 20, VI 22, X 41).

Electron micrographs of perinuclear fragments of the cytoplasm of experimental cells show greater number of elongate, transversely striped bodies, the trichocysts (Pl. II 6, VI 22, X 41). It is due to the fact that during UV irradiation the ciliates are pressed between two quartz slides of the rotocompressor in order to be immobilized (Fokin and Ossipov 1975). This procedure causes shotting off the trichocysts to outside as well as to inside the cell.

Series 2. Important ultrastructural changes are transferred with the generative nucleus to the offspring of the ciliates fixed 2 days after UV irradiation. In sections the Mi forms numerous lobes (IV 15, V 17, 18). In comparison with control ones (I 2, V 19) the chromatin granules (60 nm in the diameter) significantly increase in size aud, as a rule, occupy the central position in the Mi (IV 15). The Mi caryoplasm

is filled with soft, fine fibrillar matrix (V 18). In the phase corresponding to metaphase (Lewis et al. 1976) are visible (V 20) the condensed chromatin elements inside the funnellike structures from which the microfilaments get off joining together near the nuclear envelope (thickness of NE — 20.2 nm).

Bunches of condensed, anastomozed chromatin threads (103 nm in the diameter) are evenly dispersed in the whole Ma making it extremely similar to the Ma in the series 1. However, the NE (20.5 nm thick) forms more pronounced invaginations producing well developed lobes (Pl. IV 14, 16).

The cytoplasm contains elements of the endoplasmic reticulum (Pl. V 20).

Series 3. As a rule, the Mi in postirradiation offspring of ciliates, fixed 6 days after the beginning of the experiments, are lobed (Pl. VI 22, 26). Large, compact chromatin threads are concentrated in the central part of the nucleus (Pl. VI 21, 25). In the periphery (NE 20.2 nm thick), as well as in the centre of the Mi, numerous bunches of microfilaments occur (Pl. VI 21, 26). However, in this series of experiments the generative nuclei may also be found having less dense chromatin elements evenly dispersed in the nucleus and situated in funnels formed by microlamellae (microseptae periodicity 11.2 nm) and connected with microfilaments (Pl. VI 26, VII 28, 30). The NE continuity in such Mi may be disturbed in some cases by disruptions (Pl. VI 22, 23, 24).

The Ma contains chromatin threads, 96 nm in the diameter, composed of tightly interlaced elementary fibrilles (Pl. VII 27). The NE, 20.7 nm thick, usually bears invaginations. In electron micrographs of this series the pores in the NE are more frequent than in the former one.

The perinuclear cytoplasm of the ciliates comprises alveoles and cisterns of the endoplasmic reticulum (Pl. VII 29).

Series 4. The Mi of ciliates fixed 30 days after irradiation is oval (Pl. VIII 33, 35) or composed of a number of lobes joined together (Pl. VIII 34). In both cases the caryoplasm, fulfilling the whole space inside the generative nucleus, occurs in form of fine fibrillar matrix. In some nuclei distinct chromatin structures are visible being distributed in disorder (Pl. VIII 33, IX 37). The microfilaments, 13 nm in the diameter, sharply stands out against an electron clear background of the caryoplasm (Pl. IX 36). Appearance of large disruptions in the NE (20.2 nm thick), through which the content of the Mi flows into the cytoplasm, seems to be important (Pl. X 37, 38). It is worthy of mention that the Mi is present practically in all the cells.

Ultrastructure of the Ma in ciliates fixed 30 days after inactivation of the Mi shows densely packed chromatin threads, 103 nm in the diameter, evenly arranged in the whole space inside the Ma (Pl. VIII 31, 35, X 39). The NE, 20.9 nm thick, forms greater number of deep invaginations lying close together (Pl. X 40). The depth of some invaginations overpasses half of the transverse axis of the Ma. Thus, large lobes are formed (Pl. X 39, 41). In some cases fragmentation of the vegetative nucleus may be seen (Pl. VIII 31). Numerous fragments of the Ma have been ob-

served by us in the light microscope investigations, in Feulgen stained preparations. In lacunes formed by envelopes of two tightly adhering fragments of the Ma occur flattened canals and cisterns of the endoplasmic reticulum (Pl. X 39, 41).

Perinuclear zones of the cytoplasm are also filled with cisterns and alveoles (Pl. VIII 32).

Series 5. In this series of experiments the nuclear apparatus in the T2UV-72-2 clone, irradiated 3 years before, has been studied. The Mi is provided with the typical, undisrupted NE (20.7 nm thick). The whole space inside the generative nucleus is filled with fine fibrillar matrix with scarce single fibrilles of DNA arranged mainly in the central zone of Mi (Pl. XI 42, 43) in which branches of microfilaments are also concentrated (Pl. XI 44, 45). Despite of extremely poor quantity of DNA in the nucleus, the microlamellae (microseptae periodicity 16.5 nm) during metaphase–anaphase stage remain connected with more condensed chromatin elements (Pl. XI 44).

The shape of the Ma is typical of *P. bursaria* having slightly incised border; fragmentation has not been observed (Pl. XII 46). Chromatin threads (102 nm in the diameter), not electron dense, are evenly arranged in the whole space of the nucleus (Pl. XII 47). The nuclear envelope, 21.2 nm thick, bears pores with the central granule or without it (Pl. XII 47, 48).

Electron clear perinuclear cytoplasm comprises small quantity of the endoplasmic reticulum elements (Pl. XII 46).

Discussion

The results of our electron microscopical investigations show that UV irradiation of the Mi causes important changes in the structure of the nuclear apparatus in *P. bursaria*. Such changes have been hitherto described only on the light microscope level (Fokin 1978 b, 1979 a). It has been assumed that the Mi disappears in the offspring of irradiated cells (during 2–3 generations) due to unequal distribution of its derivatives to daughter individuals and subsequent destruction of this nucleus. We have supposed also that the fragmentation of Ma in *P. bursaria* after irradiation of Mi is connected with possible change in differential activity of the Ma genome after the loss of generative nucleus by the cell. As a morphological premise of such change in functional activity the process of the somatic nucleus fragmentation has been considered (Fokin 1978 a).

Ultrastructural investigations of morphological changes in the nuclear apparatus following UV irradiation of the Mi have shown that the irradiated generative nucleus (expositional dose 306 J/m^2) is preserved in postirradiation offspring up to 1 month and longer. However, the structure of the chromatin elements in such Mi undergoes important changes: the longer time after irradiation of the generative nucleus to the moment of fixation of postirradiation offspring the more pro-

nounced diminution of DNA quantity is observed in the nucleus (Pl. II 6, VI 21, 22, 24, 26, VIII 33–35). Besides, the ultrastructure of Mi undergoes serious changes: the zones of compact chromatin are replaced by diffuse chromatin. Despite of extremely small quantity of chromatin (series 4 and 5), proved by the presence of funnel-like lamellar structures and microfilaments, such generative nuclei are able to divide (Pl. VII 28, IX 36, XI 44).

The contour of the nuclear envelope in sections of Ma in the cells of postirradiation offspring as early as in 2 and 6 days sharply differs from that in the control ones: the nuclei are frequently multilobular (Pl. V 17, VIII 34) and the NE of Mi shows disruptions (series 3 and 4). However, such disruptions do not cause, probably, elimination of the nucleus. The results of experiments (series 5) show that in the T2UV-72-2 clone discharging of the nuclear material through the disruptions of NE into the cytoplasm does not lead to final destruction of the generative nucleus.

Thus, the conclusions about extremely high (in comparison with other species of paramecia) vitality of the Mi$^-$ subclones of *P. bursaria*, drawn earlier from the light microscope observations (Fokin 1979 b), ought to be verified. The electron micrographs obtained by us have shown that the cells of the T2UV-72-2 clone (3 years after irradiation) have the Mi, although containing extremely small quantity of the chromatin dispersed in the whole space in the nucleus (XI 40–43). Thus, they can not be regarded as genuine Mi$^-$ type. In such nuclei the chromatin can not be revealed by common cytochemical methods, such as Feulgen reaction and rivanol-SO_2. The high vitality of UV subclones of *P. bursaria*, up to 75% at least in a series of cases (Fokin 1978 a), seems to be the result of the presence of strongly changed but functioning nucleus. The electron microscopical data do not exclude the probability of the lack of Mi in some cells, however, we have no observation on the ultrastructure of cells really deprived of the Mi.

According to formerly published data (Fokin 1978 a, Fokin and Ossipov 1981) the existence of natural Mi$^-$ populations of *P. bursaria* (cf. Golikova 1978), long time without generative nuclei, is possible due to the change in the activity of Ma genome. However, ascertainment of a real amicronucleality is a difficult problem (Skoblo et al. 1978). For example, in *P. caudatum* a hopeful method of revealing the Mi, also with insignificant quantity of DNA, is infection of the nucleus with a specific symbiont, *Holospora undulata* (Rautian et al. 1975, Ossipov et al. 1976, Fokin and Borchsenius 1978). In other species of paramecia such methods have not yet been elaborated so the data on the lack of Mi in vegetative cells ought to be taken with caution.

Recognition of the nuclear apparatus of ciliates as a single functioning system (Fokin and Ossipov 1981) allow to understand the reaction of the vegetative nucleus against impairment of the Mi as quite normal. Fragmentation of the Ma following Mi irradiation or mechanical impairment was observed by us in three species of paramecia, in *P. bursaria*, *P. putrinum* and *P. caudatum* (Fokin 1978 a, 1980), the most frequently (up to 45% of operated cells) being noted in *P. bursaria*.

Such reaction of the Ma is either due to substantial loss of the Mi or due to symbionts harboured by this nucleus (Golikova 1978).

The results of observations on the dynamics of the appearance of fragments of the Ma are also given in the present paper. Deep invaginations in the nuclear envelope and lobe formation in the Ma began in *P. bursaria* as early as in 0.5–7 h following Mi irradiation. Within 2 days and, the more, within 6 days after inactivation of the generative nucleus development of large lobes was observed in the Ma (Pl. IV 14, 16). Thirty days later the fragmentation of the vegetative nucleus was clearly expressed (Pl. VIII 31). Later on this process was stopped. In the cells of the T2UV-72-2 clone, fixed 3 years after Mi irradiation, the fragmentation did not occur. According to the light microscope observations complete disappearance of the nuclear fragments in the cells of this clone takes place 2 months after inactivation of the Mi. Similar data on long continuance of the Ma fragmentation have been noted in *P. putrinum* (Fokin 1979 a). Thus, similarly to *P. putrinum*, a defined relation exists between fragmentation of the vegetative nucleus and local impairment of the Mi, the loss of the generative nucleus is not indispensable.

Impairment of the Mi leads to its morphological change, being not connected with complete ceasing of its function. Nevertheless, the possibility of change of the functional state of the Mi after irradiation can not be excluded. The evidence for this gives the change of the chromatin structure in the Mi, expressed by its decondensation. Increasing surface of the nuclear envelope of the Ma due to formation of numerous fragments involves the increase of the number of pores (series 3 and 5) considered as a manifestation of metabolic processes intensification (Čencov and Poljakov 1974). No visible changes in the ultrastructure of the chromatin of the Ma and its fragment have been observed during the experiments. According to our supposition such change is possible in the case of complete loss of the Mi. In such situation, probably, premises arise for pseudo-Mi that is Feulgen-positive bodies formation, resembling the Mi and separating from the Ma by cutting off portions of the nuclear material to the cytoplasm. According to some observations by their function these structures perform the role of the lost generative nuclei, changing into pseudomicronuclei (Ammermann 1970).

In our opinion the structure of the perinuclear zone of cytoplasm is worthy to be mentioned and discussed in connection with fragmentation of the Ma. We have found that parallelly to increasing number and enlarging lobes of the Ma the perinuclear zone of the cytoplasm contains more and more alveoli and cisterns of the reticulum (Pl. VII 29, VIII 32). The more so, in narrow spaces between the lobes greater aggregations of endoplasmic reticulum elements may be observed (Pl. X 39, 41).

The mode of "outbuilding" of the nuclear envelope, due to Ma fragmentation, remains unknown. However, it seems, that the alveoli and separate closed cisterns concentrated in the cytoplasm represent a deposit of the material need for enlarging nuclear envelope.

Some facts are known in the literature on the occurrence of intranuclear store of membranelles which are utilized during sharp changes of the nucleus capacity, i. e., in *P. bursaria* during infection by symbiotic bacteria, *Holospora acuminata* (Ossipov et al. 1980). The activity of membranes manifests itself in formation of membranaceous folds in spermatocytes of *Succinea putris* during the mitotic prophase (Danilova and Šilejko 1978), or during disintegration of the nuclear envelope of SPEW cells during mitosis (Začepina et al. 1976), etc. Not less important are the data showing that the dynamics of changes in the nuclear envelope may be connected with endoplasmic reticulum elements. Thus, during fragmentation of poliploid trophoblasts of rats narrow spaces of the cytoplasm, fulfilling deep folds of the nuclear surface, contain numerous canals and cisterns (Zybina 1979, 1980 a, b). In conjugating *Didinium nasutum* during the process of Ma desintegration the cisterns originating from the nuclear envelope have been found in cell cytoplasm, sometimes being arranged in circles (Karadžjan and Raikov 1977). During conjugation of *Bursaria truncatella* numerous alveoles of multilayer membranaceous origin, cut off from the Ma were observed (Sergeeva 1976). Of course, independently of the nature of these membranaceous structures, intra- or extranuclear, they are present in cells as a kind of deposition, realization of which takes place in a defined stage of cell activity.

Thus, local irradiation of the Mi with preservation of the generative nucleus, leads to changes in chromatin structure as well as in configuration of the nucleus. For the complete elimination of the Mi in *P. bursaria* with the aid of UV rays a dose greater than 306 J/m^2 is needed and the lack of the nuclei must be carefully checked. Even greatly changed Mi, with very low quantity of DNA, being present in a series of generation, proves its significance for living activity of vegetative cells. It is worthy of mention that the polyploid generative nuclei of paramecia (*P. caudatum*, *P. bursaria*, *P. putrinum*) are able to survive in many generations even if they contain low quantity of DNA in comparison with wild type (Jankowski 1972, Golikova 1978, Fokin 1978 a). Cells provided with such Mi show normal fission rate and surviving ability (Fokin 1978 a) as well as reorganization of the nuclear apparatus in the process of conjugation (Skoblo and Borchsenius 1979). Thus, it is evident that much lower quantity of DNA is sufficient for the Mi to perform its function than that occurring in the wild type.

The results of the action on the nuclear apparatus of *P. bursaria* show especially great interest when compared with the data on reduction of the definitive Ma genome in this paramecium (Schwartz and Meister 1975, Schwartz 1976). Really, the morphological reaction of the Ma against Mi irradiation is the most characteristic in this species (Fokin 1978 a, 1980). There are some mentions on pseudomicronuclei formation in *P. bursaria* (Schwartz 1958, Golikova 1978), hovever, it is not clear in what a way such pseudomicronuclei, originating from the fragments of Ma, may perform their functions. Further studies are needed to elucidate this question.

Fragmentation of the Ma in postirradiation offspring of *P. bursaria* with irradiated Mi (Fokin and Borchsenius 1978, Fokin 1979 a) shows close interaction between these heteromorphic nuclei. One of the arguments for the existence of such interaction in the paramecium cell is a phenomenon of change of the mating type in *P. caudatum* after UV irradiation of the Mi (Skoblo and Borchsenius 1979). As it is known, the mating type in ciliates is determined by functional activity of the Ma. Change of the mating type allows to suppose that the character of Ma activity has changed as a result of change of Mi activity.

The most severe consequences of disturbing of interaction between the nuclei due to local UV irradiation of the Mi may be expected on morphological as well as functional levels in the case of the loss of Mi. If the Mi persists irradiation the main changes in the Ma depend on enlargement of the surface due to invagination of the nuclear envelope and fragmentation and probably, on growing number of pores. Thus, the above particularities give the foundation for a supposition about the change of activity of the Ma following Mi irradiation.

The following hypothetical scheme of interaction between the nuclei of *P. bursaria* after UV irradiation is proposed. During the period of the loss of the most part of DNA by the micronucleus (a long process according to our observations) the macronucleus desintegrates into fragments enlarging thus its surface. It is possible that due to compensatory mechanism of the whole metablism of the cell the spectrum of gene activity of the Ma also changes (e. g., the intensity of DNA synthesis). After stabilization of the structure of Mi (from 30 days up to 3 years after irradiation according to our data) the process of Ma fragmentation is stopped, and the changed Mi appears to be able to perform its functions.

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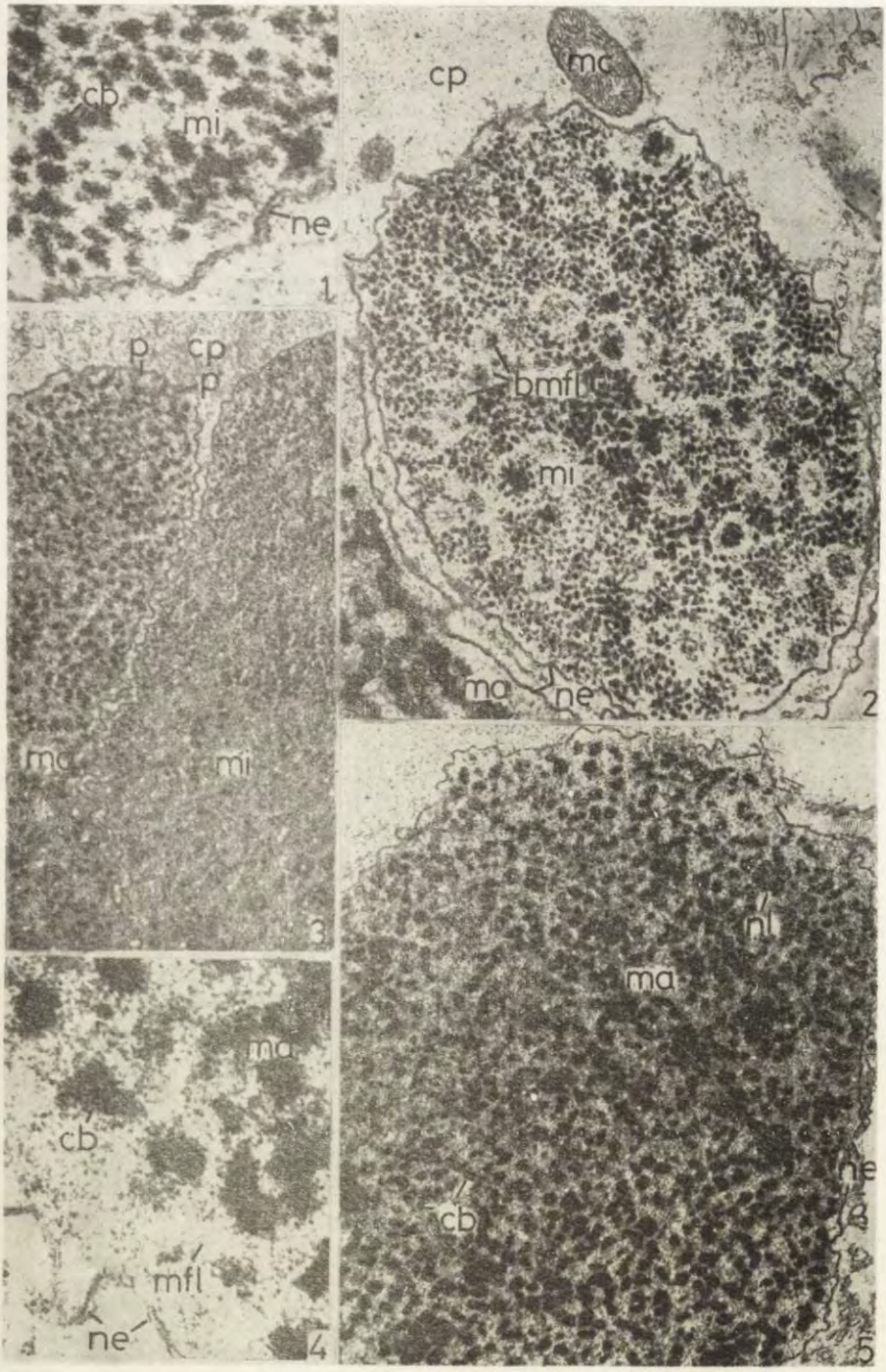
EXPLANATION OF PLATES I–XII

1–5: Electron micrographs of the nuclear apparatus in *Paramecium bursaria*, clone BP80. C ontro
1: Fragment of the micronucleus, 75 000 ×
2: Fragments of the macro- and the micronucleus and of the cytoplasm, 22 000 ×
3: Macro- and micronucleus, 13 000 ×
4: Fragment of the macronucleus, 50 000 ×
5: Macronucleus, 15 000 ×
6–9: Electron micrographs of the structure of the micronucleus in cells of the clone BP80, 0.5–7 h after irradiation
6: Micronucleus — funnel-like structures with particles of condensed chromatin elements inside, 57 000 ×
7: Mitochondria, 45 000 ×
8: Fragment of the micronucleus, 93 000 ×
9: Fragment of the cell — the macro- and the micronucleus, 27 000 ×
10–13: Electron micrographs of the structure of the macronucleus in cells of the clone BP80, 0.5–7 h after UV irradiation of their micronucleus
10: Macronucleus and the cytoplasm with elements of endoplasmic reticulum, 75 000 ×
11: Invagination of the nuclear envelope of the macronucleus, 22 000 ×
12: Fragment of the macronucleus — inner fold, 75 000 ×
13: Fragments of the macro- and the micronucleus, 75 000 ×
14–16: Electron micrographs of the nuclear apparatus in the offspring of cells, BP80 clone, 2 days after UV irradiation of the micronucleus
14: Macronucleus — deep invagination of the nuclear envelope 57 000 ×
15: Micronucleus and a fragment of the macronucleus. The contour of the nuclear envelope of Mi and centrally situated chromatin structures are visible, 13 000 ×
16: Lobes of the macronucleus, 27 000 ×
17–20: Electron micrographs of the structure of the micronucleus in the offspring of cells, BP80 clone, 2 days after irradiation of Mi
17: Cross section through numerous lobes of the micronucleus, 27 000 ×
18: Micronucleus — chromatin granules evenly dispersed in the caryoplasm, 23 000 ×
19: Chromatin granules in the micronucleus, 75 000 ×
20: Micronucleus — funnel-like structures filled with condensed chromatin elements, 24 000 ×
21–26: Electron micrographs of the structure of the nuclear apparatus in the offspring of cells, BP80 clone, 6 days after UV irradiation of Mi. Arrows show places of disruption of the nuclear envelope
21: Micronucleus — chromatin elements situated in the central part, 12 000 ×
22: Cross section through the multilobular micronucleus, 26 000 ×
23: Fragment of disrupted nuclear envelope of the micronucleus, 100 000 ×
24: Micronucleus and a fragment of the macronucleus, 51 000 ×
25: Fragment of the micronucleus — chromatin threads, 75 000 ×
26: Multilobular micronucleus — numerous bunches of microfilaments, 26 000 ×
27–30: Electron micrographs of the structure of the nuclear apparatus in the offspring of cells, BP80 clone, 6 days after UV irradiation of Mi
27: Fragments of the macronucleus and of the cytoplasm, 75 000 ×
28: Fragment of the micronucleus with funnel-like structures, 57 000 ×
29: Fragment of the cytoplasm with elements of the endoplasmic reticulum, 75 000 ×
30: Micronucleus — numerous bunches of microfilaments are visible, 57 000 ×
31–35: Electron micrographs of the structure of the nuclear apparatus in the offspring of cells, BP80 clone, 30 days after UV irradiation of Mi
31: Fragmented macronucleus, 13 000 ×
32: Fragments of the cytoplasm and of the macronucleus, 57 000 ×
33: Micronucleus showing the caryoplasm filled with fine fibrillar matrix, 15 000 ×
34: Multilobular micronucleus, 12 000 ×
35: Micronucleus and a fragment of the macronucleus, 30 000 ×
36–38: Electron micrographs of the structure of the micronuclei in the offspring of cells, BP80 clone, 30 days after irradiation of Mi. Arrows indicate places of disruption of the nuclear envelope
36: Micronucleus — numerous microfilaments are visible, 57 000 ×
37: Disruptions of the nuclear envelope and bunches of microfilaments, 18 000 ×
38: Fragment of the micronucleus with disrupted nuclear envelope and the nuclear content discharging into the cytoplasm, 48 000 ×

39–41: Electron micrographs of the structure of the macronuclei in the offspring of cells, BP 80 clone, 30 days after irradiation of Mi
39: Portion of the macronucleus in course of fragmentation. Elements of endoplasmic reticulum are visible in the cytoplasm between fragments of Ma, 62 500 ×
40: Envelope of the macronucleus with numerous invaginations, 36 000 ×
41: Large lobes of the macronucleus, 13 000 ×
42–45: Electron micrographs of the micronuclei in cells of T2UV-72-2 clone, 3 years after irradiation
42, 43: Micronuclei with bunches of microfilaments, 26 000 × and 22 000 × resp
44: Funnel-like structures in the micronucleus, 75 000 ×
45: Bunches of microfilaments, 75 000 ×
46–48: Electron micrographs of the macronuclei in cells of T2UV-72-2 clone, 3 years after irradiation of Mi
46: Macronucleus, 22 000 ×
47, 48: Fragment of the macronucleus. Tangent section through the nuclear envelope, 45 000 × and 75 000 × resp

Abbreviations used:
bmfl — bunches of microfilaments, c — condensed portions of chromosomes, cb — chromatin bodies, ch — chloroplast, cl — canal of endoplasmic reticulum, cp — cytoplasm, fv — food vacuole, ma — macronucleus, mc — mitochondria, mfl — microfilaments, mi — micronucleus, ne — nuclear envelope, nl — nucleole, p — pore, t — trichocyst, tn — cistern of endoplasmic reticulum



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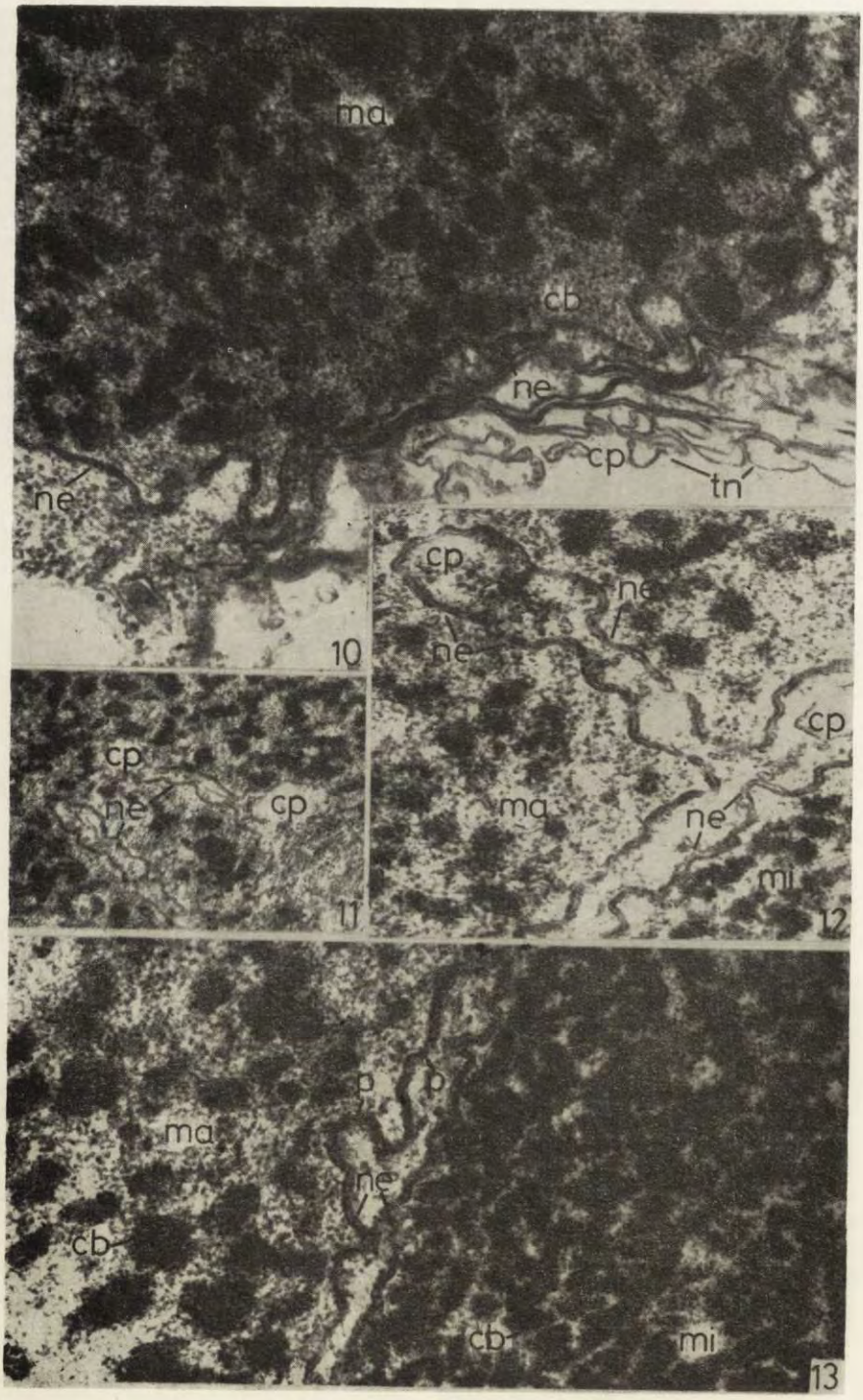

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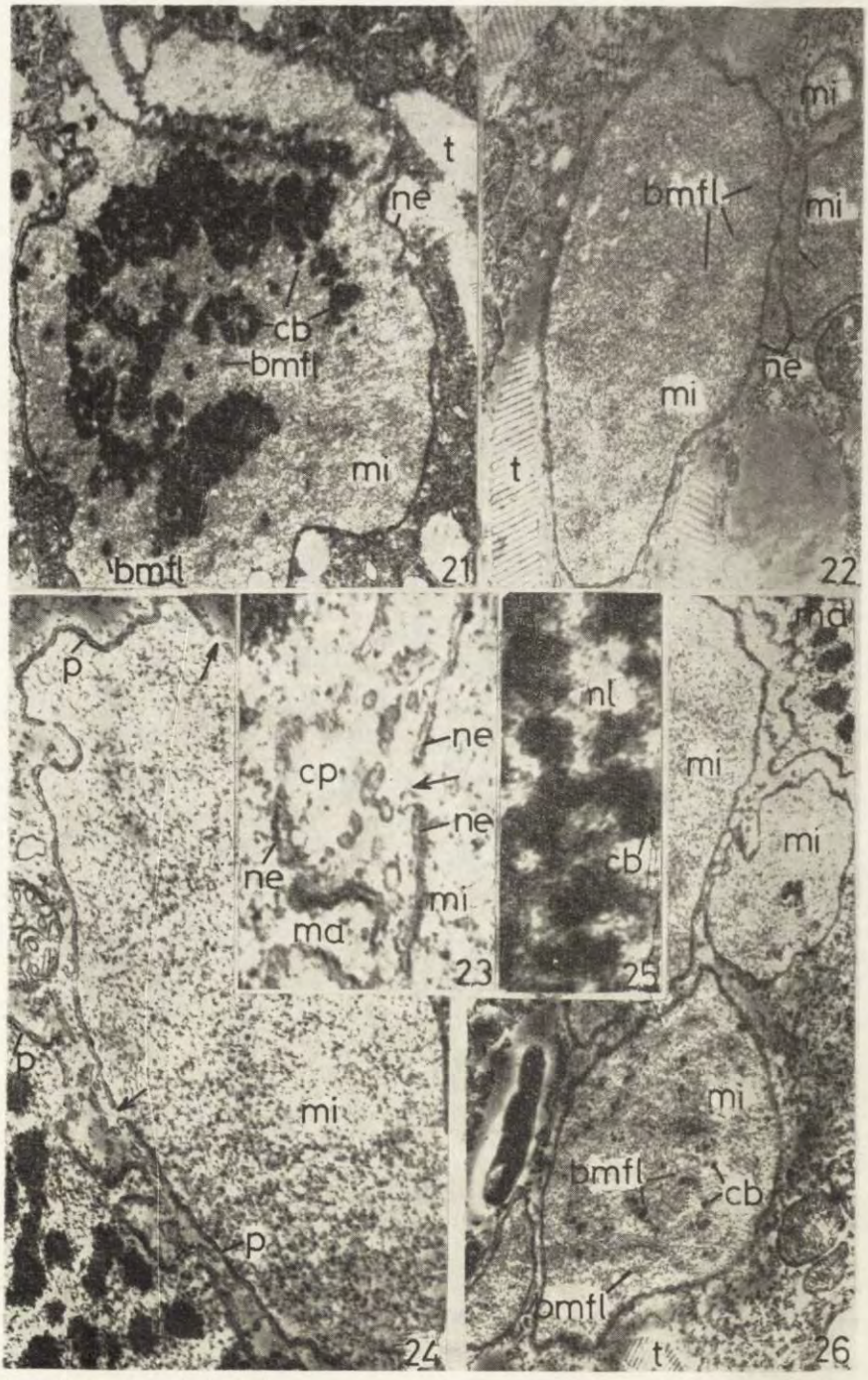

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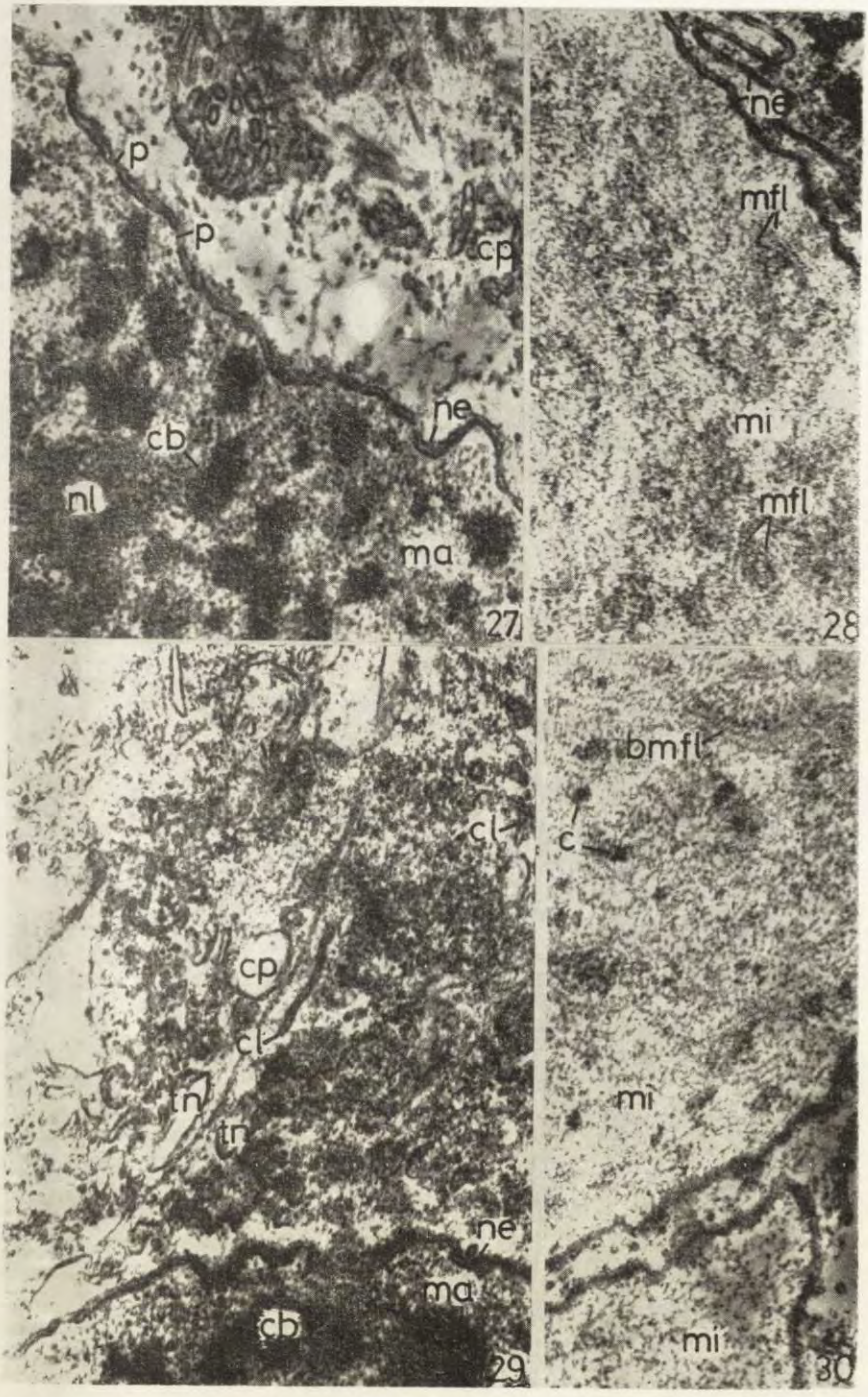

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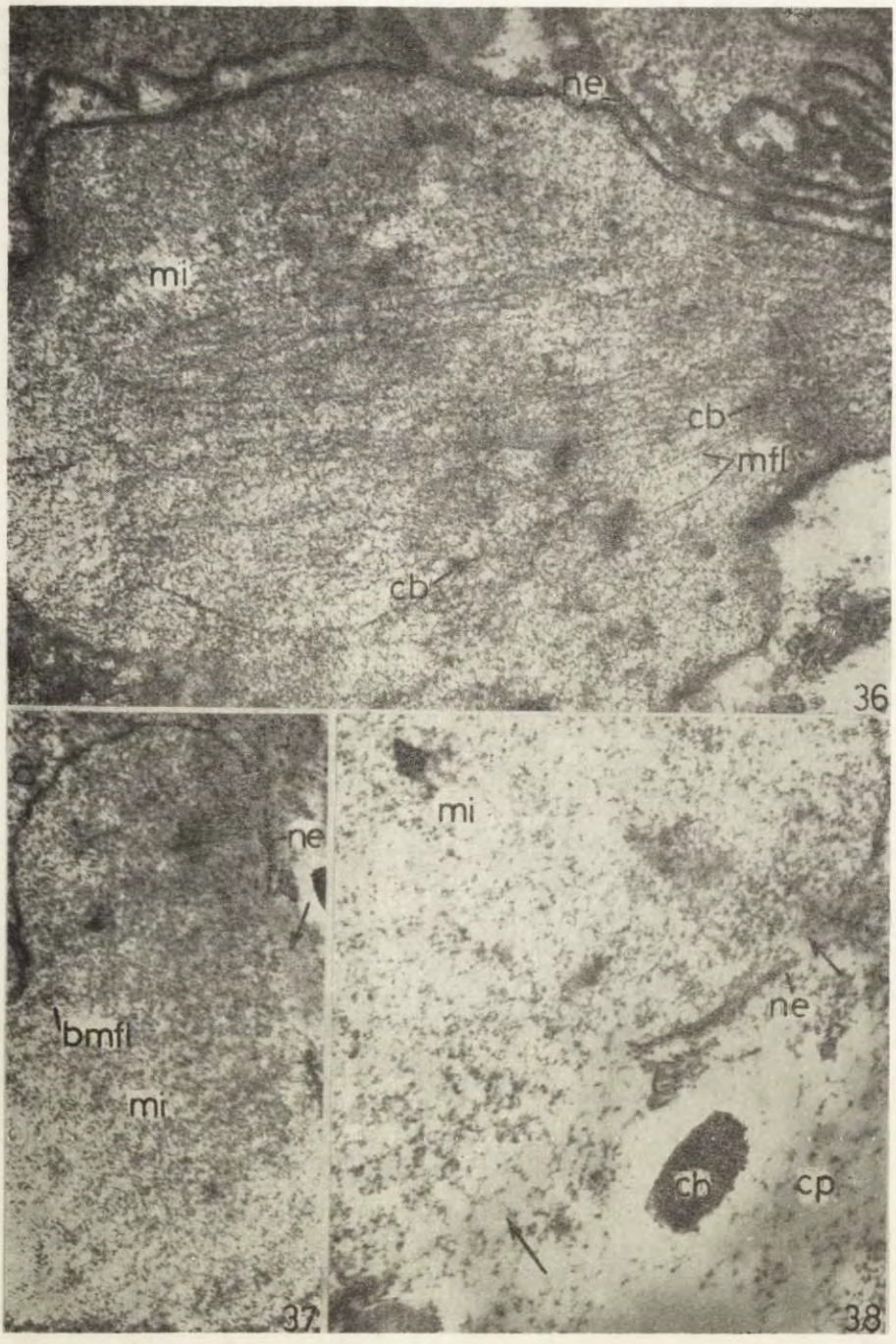

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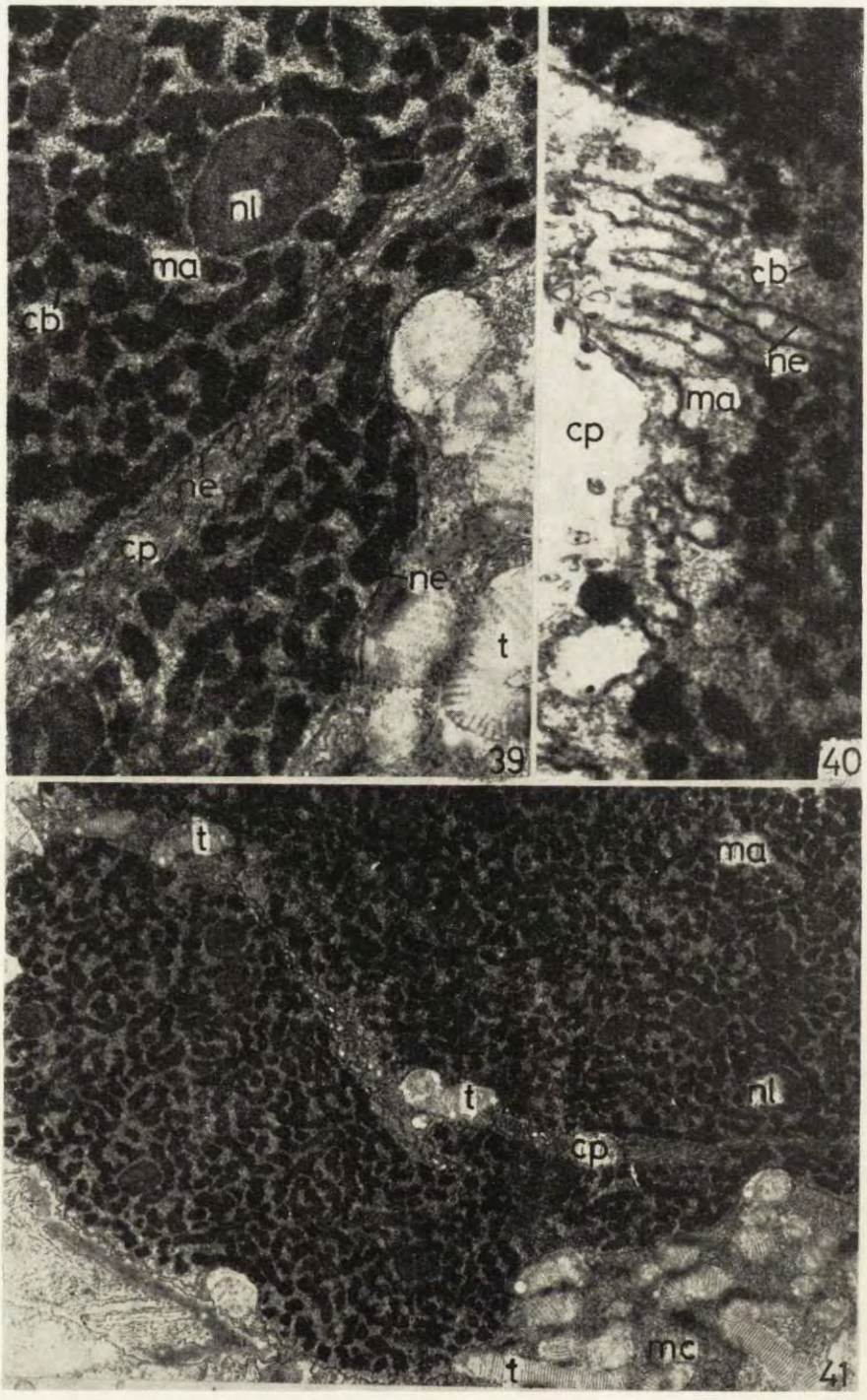

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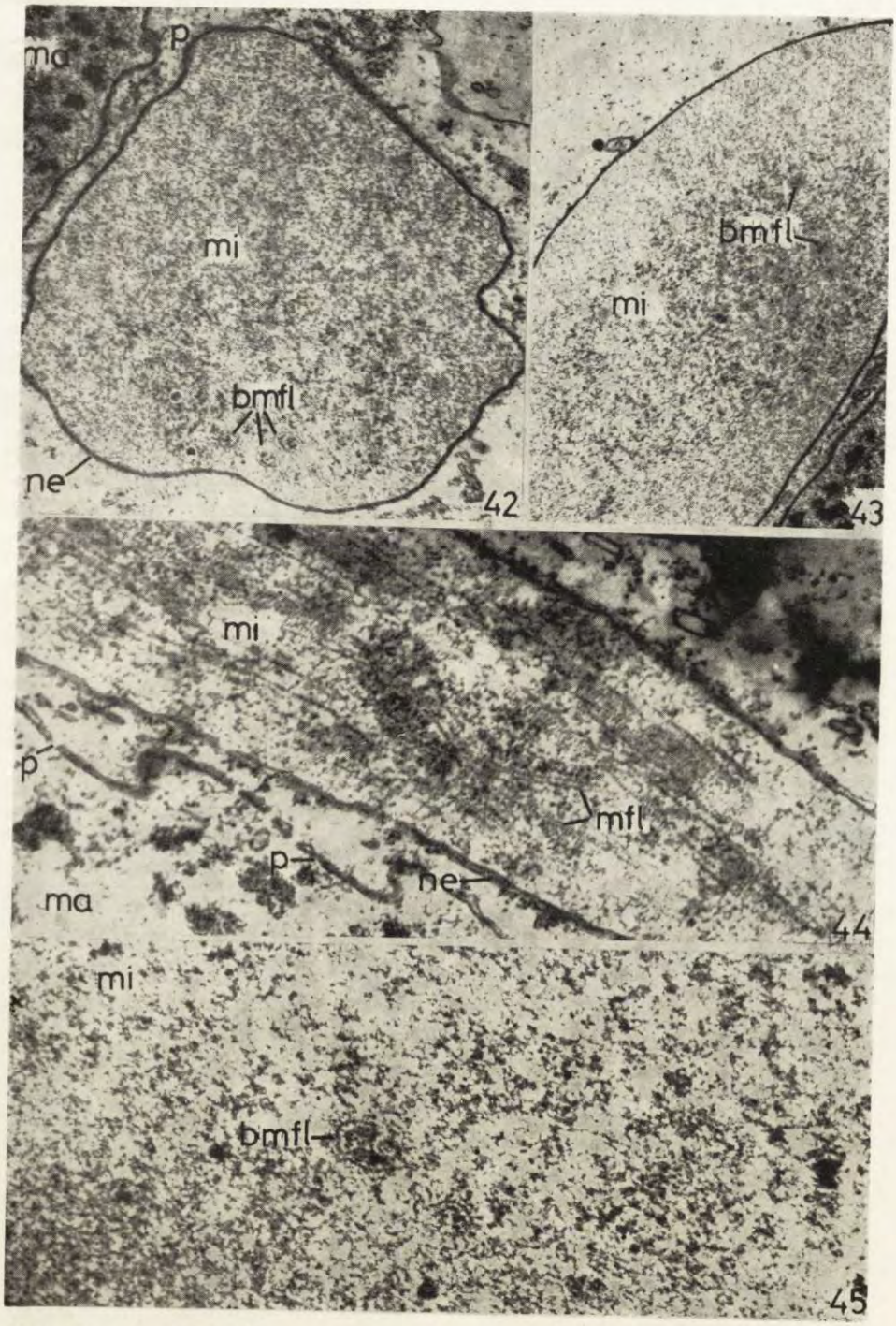

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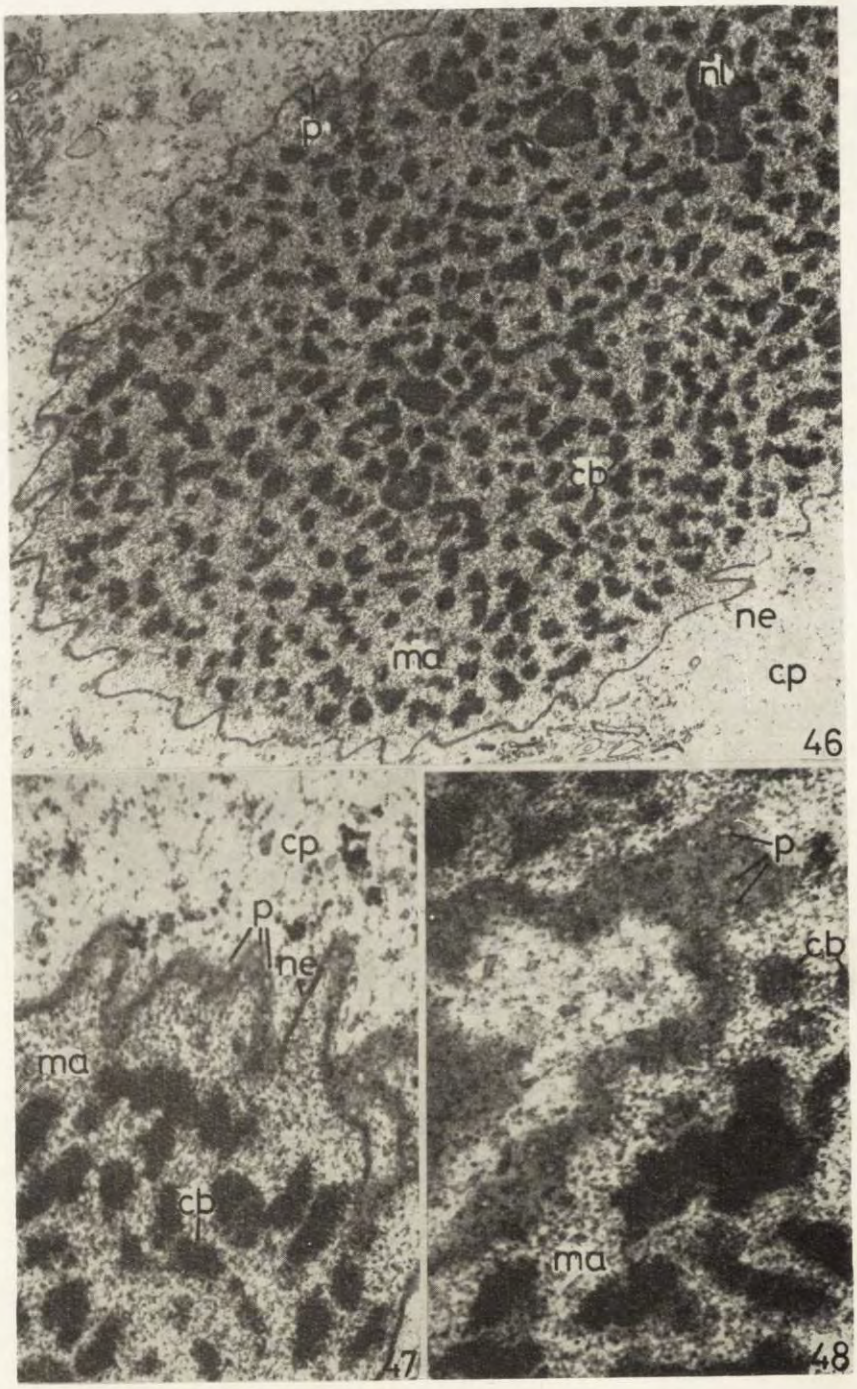

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Further Studies on the Relation between Contraction and Streaming Oscillations in the Plasmodial Veins of *Physarum polycephalum*

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Synopsis. There is a distinctly significant positive correlation in time between the contraction phase in the principal plasmodial veins and the forward direction of streaming inside them. This correlation becomes negative for the brief periods when the vein temporarily grows up. Normally, in the central regions of the network the forward streaming phase begins slightly earlier than the contraction phase. The forward streaming phase is significantly longer than that of the reverse flow (55 : 45). Application of a positive stimulus to the front of plasmodium (its lower illumination) contributes to the further increase of the forward streaming time in veins, the higher frequency of their contraction cycles, and the better synchronization between contraction and streaming oscillations.

It was recently found by several authors that in the veins isolated from plasmodia of *Physarum polycephalum* for the tensiometric measurements, the contraction pulses become synchronized along the whole length of the excised fragment (Takeuchi and Yoneda 1977, Krüger and Wohlfarth-Bottermann 1978, Yoshimoto and Kamiya 1978 a, Achenbach and Wohlfrath-Bottermann 1980 a and b). It was demostrated in parallel that in a complete intact plasmodium freely migrating on the substratum, the contraction–expansion rhythm is synchronous in the whole network of veins behind the advancing front (Grębecki and Cieślawska 1978, Cieślawska and Grębecki 1979 a and b, Hejnowicz and Wohlfarth-Bottermann 1980).

There are no methods available to map the topography of the oscillations of shuttle streaming over the whole plasmodium, as it is done for the contraction rhythms. It was only demonstrated that in two interconnected neighbour veins within the network investigated *in situ*, the synchronization of streaming oscillations is less precise than the synchronization of contraction pulses (Grębecki and Moczoń 1978, Grębecki 1979 b). Nevertheless, the contraction phases are in general expected

to be positively correlated in time with the periods of endoplasm flowing in the forward direction. This presumption is based on the finding of Grębecki and Cieślawska (1978) that contraction of the whole system of veins coincides in time with the expansion phase of the frontal edge. Grębecki and Moczoń (1978) were able to confirm the existence of such a positive correlation, but it was much less significant than it might be expected according to the mechanism of locomotion proposed by Grębecki and Cieślawska (1978).

It was decided therefore to test again the time relations between the contraction and streaming periods after slightly modifying the methods. The present investigations were limited to the thickest principal veins of plasmodium, in order to avoid local irregularities which are more likely to occur among the anastomosing secondary veins. The former microcinematographic technique was replaced by recording *in vivo* with help of the TV equipment, which allowed to work under much more appropriate light conditions.

Material and Methods

Plasmodia of *Physarum polycephalum* were grown on the wet filter paper and fed on oatmeals, according to the method of Camp (1936). Their fragments were transferred on the non-nutritive 1.5% agar gel and kept for 24–36 h in darkness. The re-organized starving plasmodia with well differentiated frontal zones and networks of veins, were used for experiments. They were placed on the stage of an inverted microscope and illuminated with the vertical white light beam (700–1400 lux) from a photographic enlarger. The enlarger illuminates evenly the whole surface of a plasmodium, whereas its illumination by the standard lighting system of the microscope is limited approximately to the dimensions of the field of view. Such uneven illumination of plasmodium may disturb its motor behaviour.

The microscope was equipped with the TV camera. The contraction–relaxation cycles of the investigated vein were automatically recorded from the TV screen by a photoelement. At the periods when the endoplasm was moving in the frontal direction, a low amplitude oscillation of 1Hz was transmitted by hand operation to the recording system. It changed the thickness of the respective segments of the pulsation curves (cf. Figs. 2, 3). At the moments of streaming reversal and at the periods of its occasional cessation, a supplementary potential difference was added to the input of the recorder. It resulted in producing vertical streaks in the case of each instantaneous change of the direction of flow, or the gaps in the recorded curve when the endoplams remained for a longer time in the resting condition (more exactly, the recorded curve was in such cases shifted a few centimeters up, as shown in Fig. 13).

First observations were made on intact plasmodia, at three control sites along the main veins and in three types of the secondary veins inside the network. Other experiments were run on operated plasmodia in which the investigated segment of the main vein remained as the unique functional channel between the posterior regions and the front, all the other interconnections being sectionned. In two last groups of experiments the effect of amputation of the front, or of its positive stimulation by shade, were examined.

The degree of synchronization between the contraction–expansion cycles of the veins and the shuttle streaming oscillations in them, was investigated by means of the correlation analysis. The time relationships between the contraction phase and the forward streaming period were checked

in each vein by the χ^2 test and expressed in form of the correlation coefficient $\varphi = \sqrt{\chi^2/N}$. Each experiment lasted 1000 s and the behaviour of the vein was controlled every 5 s. Therefore, $N = 200$, and the coefficient $\varphi \geqslant 0.18$ was significant at the $p = 0.01$ level and $\varphi \geqslant 0.12$ at the $p = 0.10$ level. The 1000 s records of the pulsation and streaming were consecutively repeated three times in the same vein. Thirty veins were investigated in each type of experiment.

Results

Observation of Intact Plasmodia

Observations were made in the thick main veins which reach 300–500 μm in diameter and connect directly the posterior regions with the front. The contraction and streaming cycles were recorded at three points along each vein: (1) at the basis of the front where the principal vein ramifies into thinner branches (site A in Fig. 1), (2) in its middle part situated in the central area of the network (M in Fig. 1), (3) in the posterior part of the principal vein (P in Fig. 1).

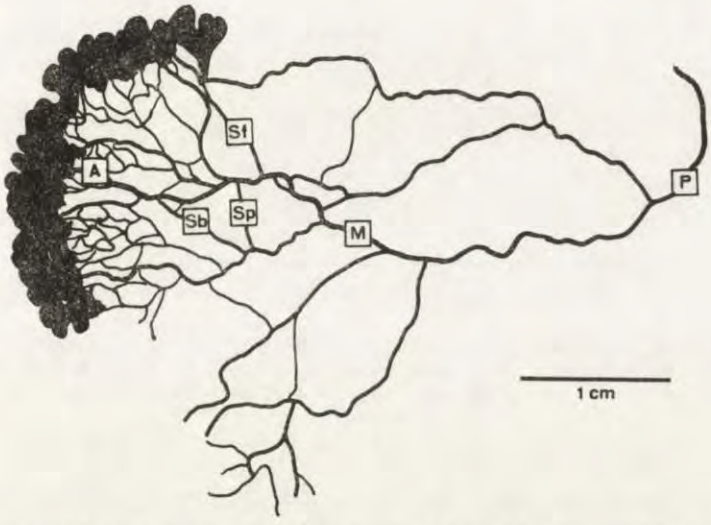

Fig. 1. Control sites in the intact plasmodium situated along a principal vein in its anterior (A), middle (M), and posterior (P) segments, and in smaller secondary veins parallel to the front (Sp), running in the frontal direction (Sf) and inclined backwards (Sb)

Typical examples of the recorded curves are shown in Fig. 2. As well the regularity of the contraction–expansion cycles as their relation to the shuttle streaming phases change along the veins length. The pulsation rhythm appears to be most stable in the posterior part of the vein (Fig. 2 c), and on the contrary, most confuse close to the front (Fig. 2 a). It is clearly seen that the periods of forward streaming strongly coincide in time with the contraction phase of the vein in its posterior frag-

ment (Fig. 2 c), this relation seems to be slightly less pronounced in its middle segment (Fig. 2 b), and rather uncertain in the vicinity of the frontal zone (Fig. 2 a). This estimation has been confirmed by the correlation analysis. The mean correlation

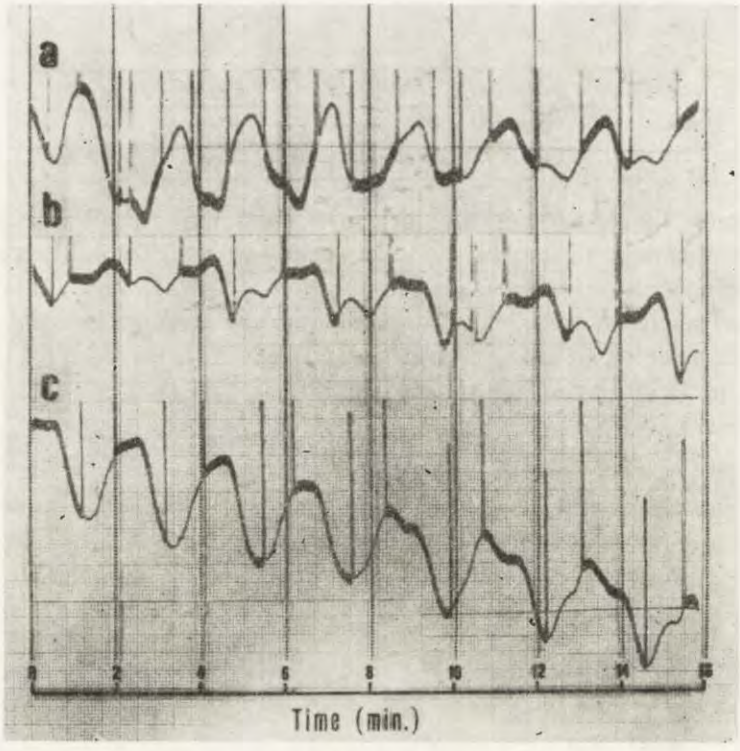

Fig. 2. Pulsation and streaming records taken in the principal vein of a plasmodium close to the front (a), in the central area of the network (b) and in its posterior region (c). Thick lines were plotted during the forward streaming periods and the thin ones when the endoplasm was flowing backwards

coefficient φ between the contraction phase and the forward streaming period calculated for 60 veins equals to 0.35 in the central area of the network, it reaches 0.42 in the posterior region, but falls down to 0.13 (which is weakly significant at the level of $p = 0.10$) close to the front.

Three other series of records were taken in thinner secondary veins inside the network. They were grouped according to the orientation of the investigated secondary branch in respect to the main vein and to the front of plasmodium: (1) secondary veins perpendicular to their respective principal "mother" veins and nearly parallel to the frontal edge (Sp in Fig. 1), (2) secondary veins deviating from the principal "mother" vein obliquely forwards in the frontal direction (Sf in Fig. 1), (3) secondary veins running from more posterior areas of the network toward the

point of their junction with the principal large vein (Sb in Fig. 1). In all these secondary veins the endoplasmic flow directed toward the main vein was conventionally treated as the forward streaming.

The curves shown in Fig. 3 demonstrate that the contraction–relaxation rhythm is most stable in such secondary veins which join the principal vein from behind (Fig. 3 b). It was found as well that in those veins the contraction and the forward

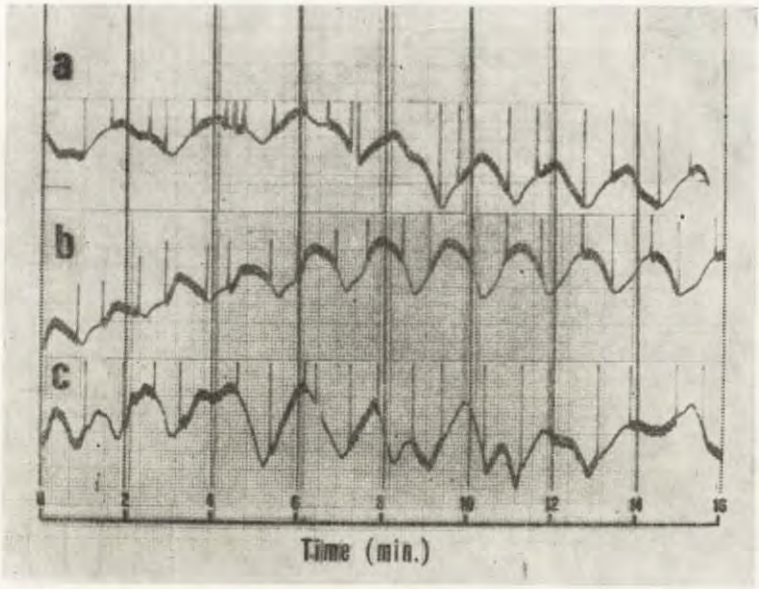

Fig. 3. Pulsation and streaming records in the secondary veins parallel to the front (a), linking a principal vein with the posterior regions of plasmodium (b) and linking it with the frontal zone (c)

streaming direction are strongly positively correlated ($\varphi = 0.37$). It means that the synchronization of contraction with the forward streaming in such secondary channels is as precise as in the principal veins of plasmodium ($\varphi = 0.35$). On the contrary, the pulsation curves appear rather chaotic in the secondary veins extended from the point of their junction with a main vein toward the front of plasmodium (Fig. 3 c). A weakly significant negative correlation was even found in them ($\varphi = -0.17$). It means that in the majority of cases the endoplasm was not squeezed during contraction toward the main vein but toward the frontal zone. In secondary veins parallel to the frontal edge the contraction phase is positively correlated ($\varphi = 0.24$) with the endoplasm streaming directed toward the junction with the principal vein. The pulsation cycles are in such veins fairly regular, although the unexpected periods of streaming cessation or even its brief reversals unrelated to the general rhythm, may be often observed in them (Fig. 3 a).

Influence of other Veins in the Network

The aim of next two series of experiments was to simplify the conditions of endoplasm flow through the investigated segment of a principal vein by reducing the probable interference of other veins interconnected in the network of plasmodium. Two types of operations were tried to achieve this goal. In the first one, all the secondary branches of the main vein situated behind the proper frontal zone were cut (Fig. 4 a), so as to separate the investigated principal vein from the network along its whole length. But the connection with the front was intact. In the

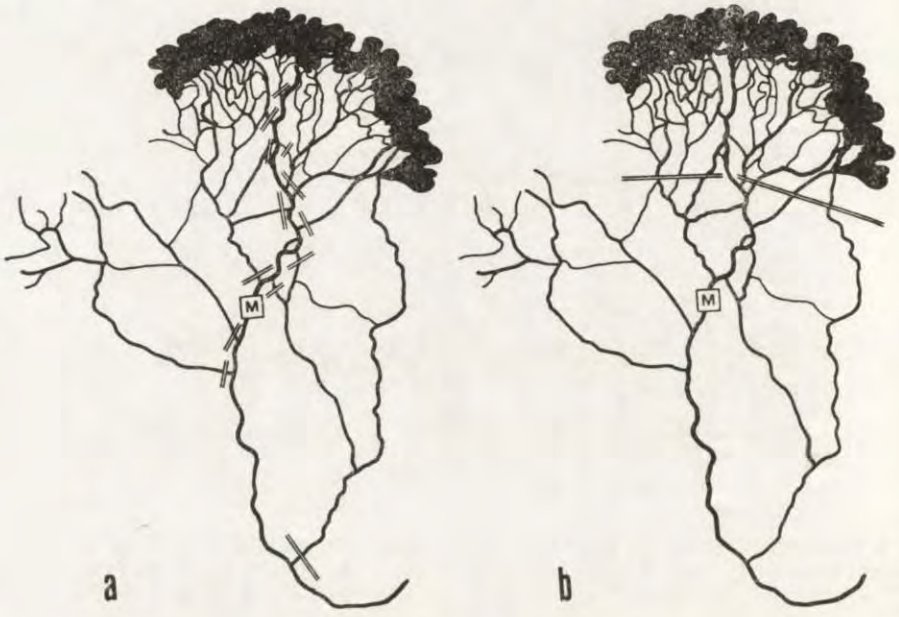

Fig. 4. Principal vein controlled at the central site M, after operation separating it from all the other veins of the network (a) or intersecting all other possible ways of the endoplasm transport toward the front (b)

second group of experiments the whole network was dissected transversally on both sides of the investigated principal vein (Fig. 4 b). It resulted in elimination of any alternative ways for the streaming. The whole mass of endoplasm transported from the posterior regions of plasmodium toward the front and back, was then obliged to pass through the vein being under control. In both experiments the first 1000 s record was taken in the intact plasmodium and the next two after the operation. The results are shown in Figs. 5 and 6.

Both types of operations gave similar results. They exerted a rather weak influence on the degree of synchronization of forward streaming with the contraction phase. The coefficient φ was only slightly increased after the operations. Their effect on

the regularity of contraction–expansion cycles was much more important, as it is clearly demonstrated by the shape of pulsation curves shown in Fig. 5 and 6. The dissections affected also the frequency of contractions: two or three cycles more might be counted in the post-operational veins along each recording period.

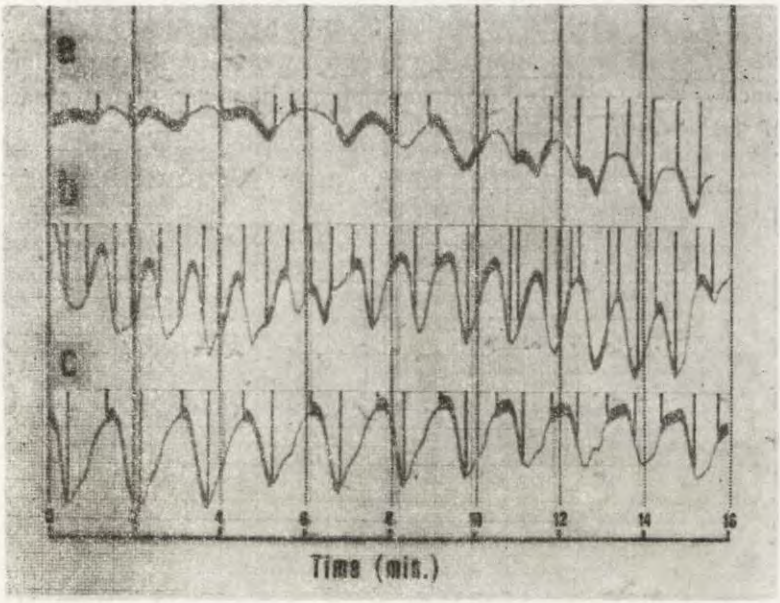

Fig. 5. Pulsation and streaming cycles recorded at the middle segment of a principal vein in the intact plasmodium (a) and after separating it from the other veins (b–c)

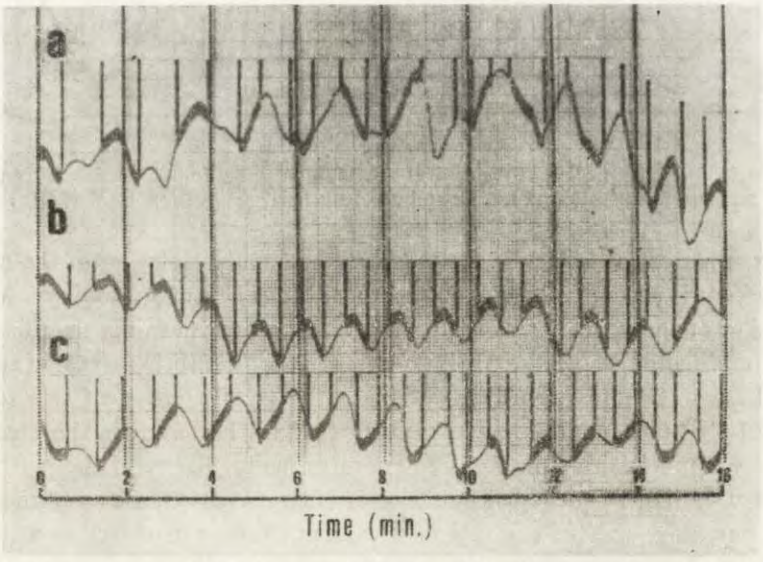

Fig. 6. Pulsation and streaming cycles recorded at the middle segment of a principal vein in the intact plasmodium (a) and after intersecting all the alternative channels of flow (b–c)

Influence of the Frontal Zone

Last two groups of experiments were undertaken to check the influence of the frontal zone upon the contraction rhythm and streaming periodicity observed in the principal veins of plasmodium. The fronts were either amputated by dissection *in situ* (Fig. 7 a) or activated by shading them with a black screen (Fig. 7 b)[1]. In both cases the pulsation and streaming direction were recorded three times in one of the principal veins: once in the untreated plasmodium, and then twice after application of the disturbing factor.

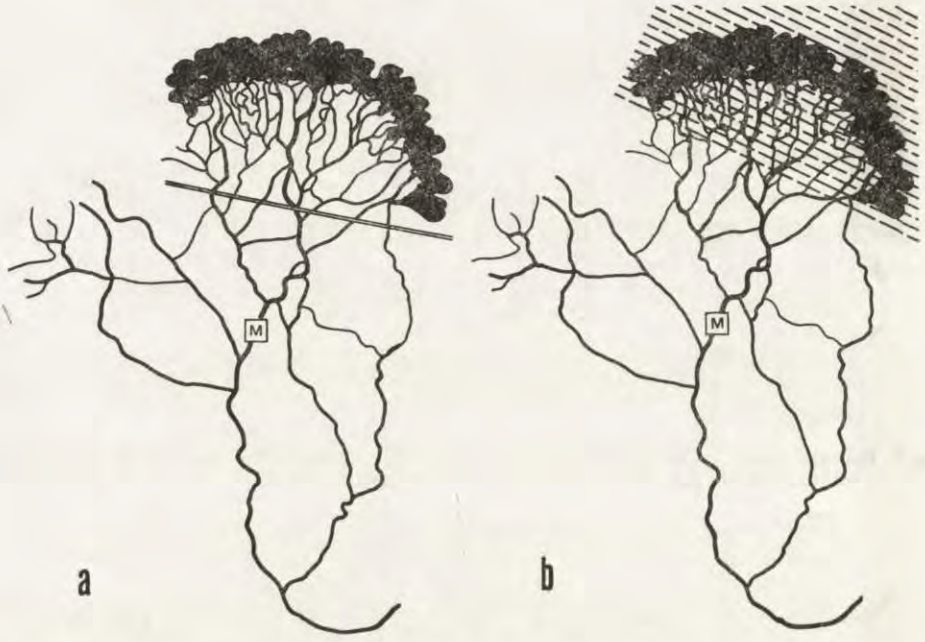

Fig. 7. Principal vein controlled at the central site M, after operation separating it from the front (a) and after activation of the frontal zone by local application of shade (b)

An example of the influence exerted by the complete isolation of the main vein from the front is presented in Fig. 8. Such operations never lead to any interruption of pulsation of the vein. It means that the vein's pulsation is the manifestation of its own contractile capacity (Hülsmann and Wohlfarth-Bottermann 1978, Grębecki and Cieślawska 1978) and not the passive effect of the activity of the frontal zone (Yoshimoto and Kamiya 1978 b). However, in the great majority of cases amputation of the front deteriorates the regularity of the motor behaviour of a vein. The frequency of pulsation is often decreased, the amplitude usually becomes unsteady, and the endoplasm streaming changes its direction at less regu-

[1] It is known (Rakoczy 1973) that the migrating vegetative stage of *Physarum* avoids light.

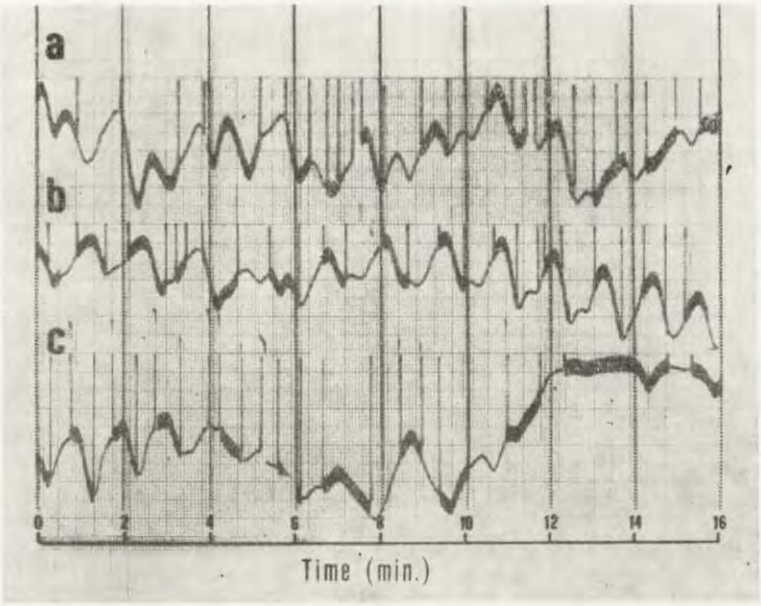

Fig. 8. Pulsation and streaming cycles recorded at the middle segment of a principal vein in the intract plasmodium (a) and after separating it from the front (b–c)

lar intervals. The operation affects most distinctly the synchronization of the forward streaming with contractions of the vein. The mean correlation coefficient φ calculated for the totality of 15 investigated cases reached 0.31 before the operation, but it was falling down to 0.13 after it.

The effects of activation of the frontal zone by shade were investigated in such veins which manifested a rather weak regularity of the contraction and streaming cycles during the first control record (Fig. 9 a). It allowed us to check whether the application of the positive stimulus to the front may improve the rhythmicity of motor behaviour in the main vein of the network. It has been demostrated (Fig. 9 b–c) that in fact the contraction–expansion oscillations become much more steady in frequency and in amplitude. Moreover, the frequency is generally increased, approximately by two cycles per 1000 s of recording. The synchronization between the contraction and streaming oscillations becomes also clearly improved. The value of the coefficient φ increases from 0.25 to 0.35.

General Relations between the Contraction and Streaming Periodicity

It has been observed in the totality of the present material that, in general, the veins which manifest more regular pulsations are also characterized by a more precise synchronization of the forward streaming periods with the contraction phases. This relation was demostrated in 60 main veins investigated at the middle of their

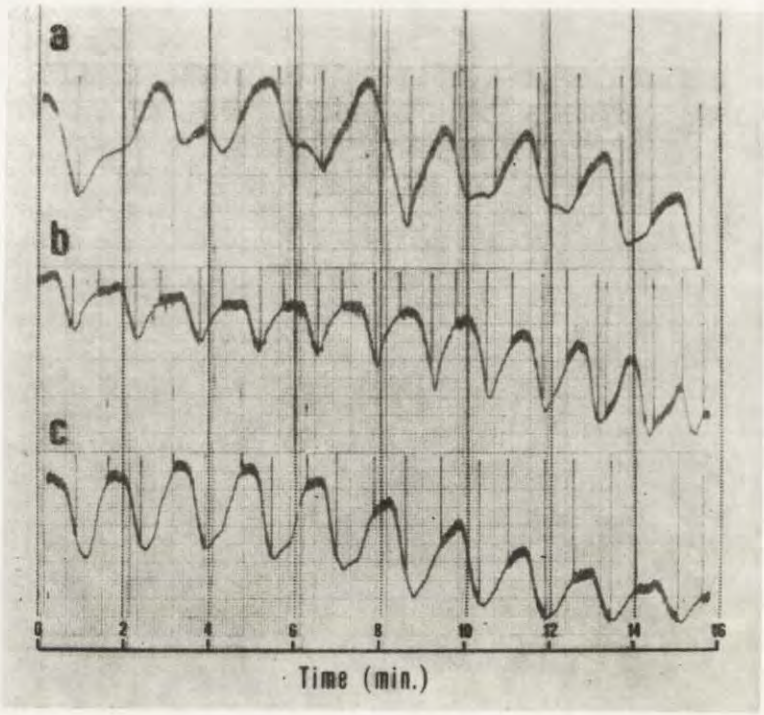

Fig. 9. Pulsation and streaming cycles recorded at the middle segment of a principal vein in the unaffected plasmodium (a) and after shading the frontal zone (b–c)

length in the intact plasmodia. Six selected curves shown in Fig. 10 are arranged in the sequence corresponding to the value of the coefficient φ which decreases from 0.65 (Fig. 10 a) to −0.01 (Fig. 10 f). It is clearly seen that the regularity of contraction–expansion cycles decreases in the same order.

The review of all regular pulsation curves presented above (Figs. 2 b–c, 3 b, 5 b–c, 6 b, 9 b–c and 10 a–d) demonstrates not only a general coincidence between vein's contraction and forward streaming, but also a very characteristic deviation from this rule. In a great majority of cases the maxima of veins pulsation (the end of expansion and the beginning of contraction) fail to coincide precisely with the moments of streaming reversal. This deviation is strictly unilateral. The endoplasm begins to flow in the frontal direction slightly earlier, before the onset of the contraction phase in the investigated area. The moment of cessation of the forward streaming and of its reversal seems to correspond much better to the terminal stage of contraction.

This phenomenon was analysed in a more detailed manner in the totality of material provided by the principal veins investigated at the middle of their length. Each individual pulsation cycle was divided in 16 fractions, 8 equal subperiods for each contraction phase, and 8 for each expansion. Then, the total number of cases

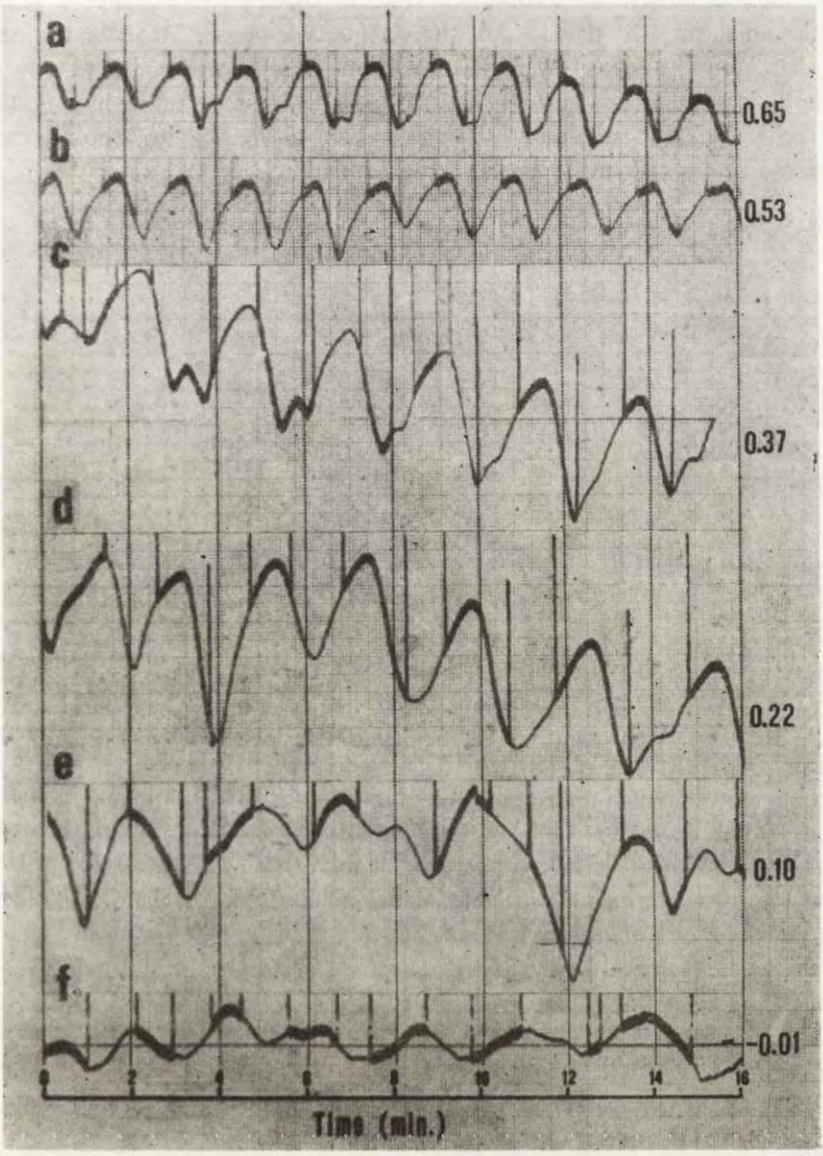

Fig. 10. Some examples of the pulsation curves recorded in the intact plasmodia arranged from a to f according to the decreasing value of the correlation coefficient φ between the contraction and forward streaming periods

of forward streaming found during each subperiod has been calculated. The results are shown in Fig. 11, in form of vertical bars of different length intersecting the record of one typical contraction–expansion cycle. The picture confirms that the frontal direction of the endoplasm flow appears and gradually becomes more and

more frequent already in the second half of the expansion phase of a vein. It reaches its maximum during the first half of the contraction phase. Then, it declines and falls rather abruptly down toward its minimum, which corresponds exactly to the minimum of the pulsation curve. It may be generally stated that in the middle part of a principal vein the streaming oscillations seem to "anticipate" slightly the contraction oscillations recorded at the same control site.

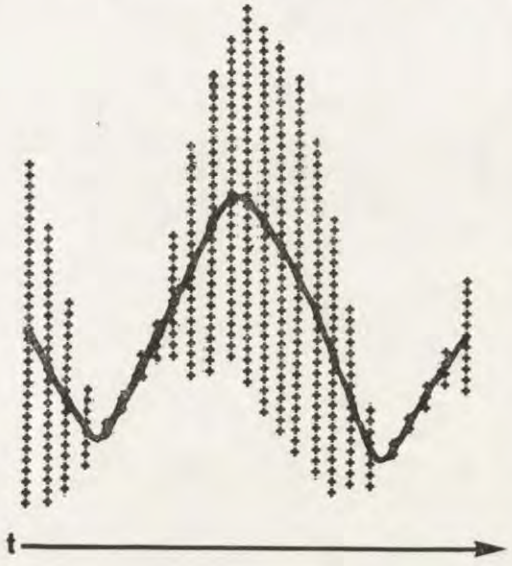

Fig. 11. Number of cases in which the forward streaming has been observed in the middle segments of principal veins, plotted against one typical contraction–relaxation cycle of such a vein

It is evident that such a situation may happen when the total time of the endoplasm streaming forwards is longer than the total time of contraction recorded in the same segment of the vein. As a matter of fact, the mean time ratio of the forward vs backward streaming calculated for all the control sites situated in the middle segments of principal veins of intact plasmodia (Fig. 1, site M) amounted to 55 : 45, whereas the ratio of contraction vs expansion time at the same control sites reached only 48 : 52.

The operations described in two preceding paragraphs had little influence on the time ratio between the two directions of streaming. But the activation of frontal zone by shade led to the increase of this ratio from 54 : 46 before stimulation up to 58 : 42 under the influence of positive stimulus.

The general coincidence of the contraction phases with the forward streaming periods is, as a rule, observed in the veins which keep their diameter approximately constant from one cycle to another, or which become gradually thinner during the recording time. When the diameter of the investigated vein gradually increases, the correlation between the contraction and forward streaming phases becomes negative, i. e., the endoplasm flows during contraction in the reverse direction. The change is very prompt and may be observed even in the case of a brief temporary increase of vein's diameter lasting no longer than for a few pulsation cycles. Such

situations are seen, for example, in Fig. 6 b between 13′ and 16′ of recording, and in Fig. 6 c between 1′ and 6′, and between 11′ and 14′. Some other examples of this phenomenon are shown in Fig. 12 (13′–16′ in a, 8′–12′ in b, and 6′–10′ in c). The change from the positive to negative correlation between the contraction and streaming cycles is achieved by a relative acceleration of the flow reversals, so that the forward streaming period temporarily covers symmetrically the maxima of the vein's pulsation (11′–14′ in Fig. 12 a, and 6′–11′ in Fig. 12 d).

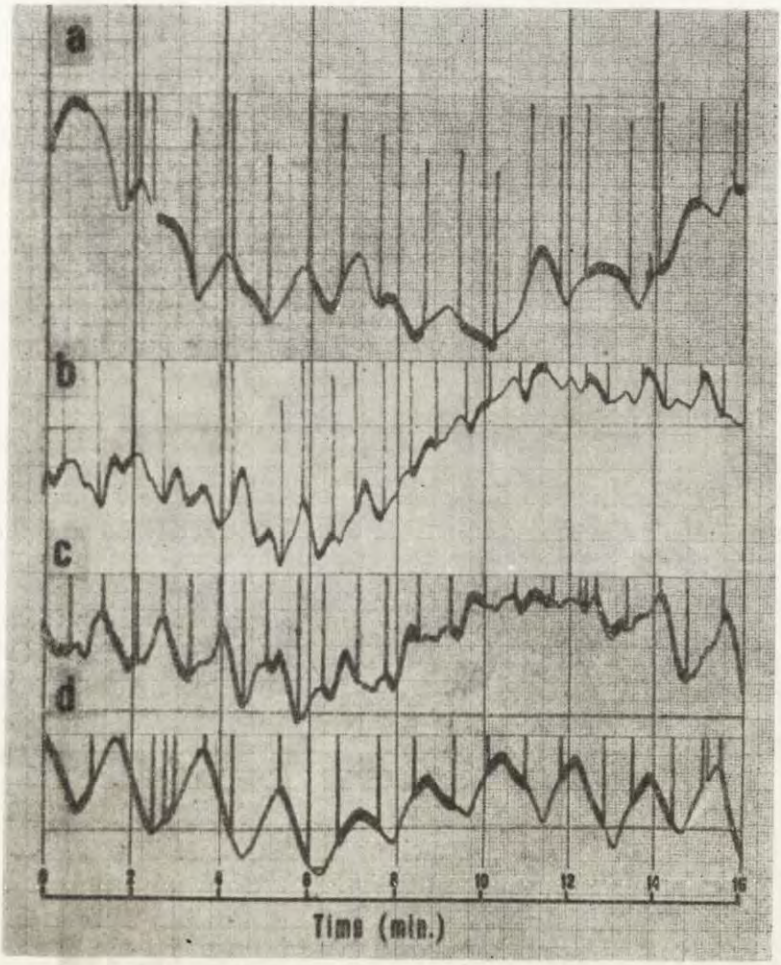

Fig. 12. Four examples of irregular pulsation cycles. Note the forward streaming periods coinciding with the expansion phase of pulsation, when the vein is generally growing up

As it was stated earlier, in some veins the shuttle streaming may be irregular and the endoplasm may sometimes come to the resting state. Three fragments of records demostrating this phenomenon are reproduced in Fig. 13. At the periods of the cessation of flow the curves of pulsation were interrupted and their missing

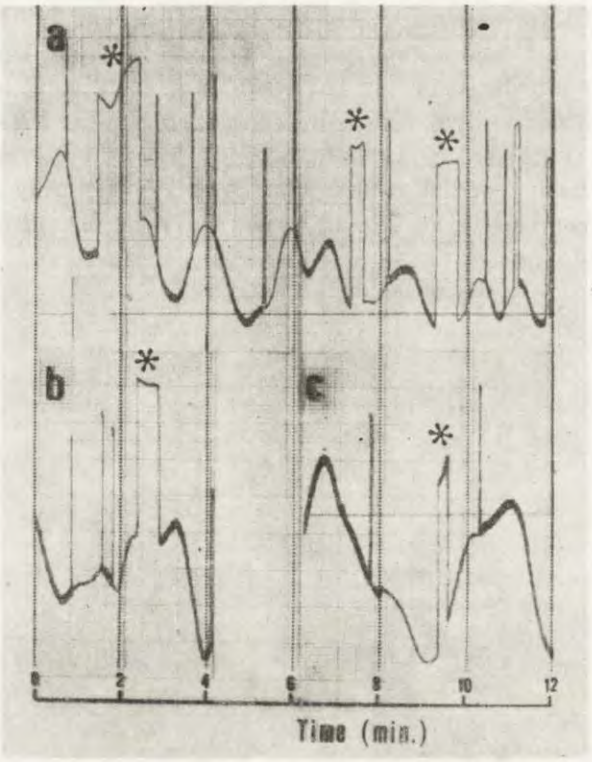

Fig. 13. Examples of the contraxion–relaxation curves in secondary veins with frequent periods of the cessation of flow. Note that at these periods the pulsation is not interrupted (asterisks)

segments were plotted by the recorder about 2 cm higher (as indicated by the asterisks). It should be noted that these segments are not flat, but they fit perfectly well within the gaps produced in the basic pulsation curve. It leads to the conclusion that a temporary cessation of flow does not disturb the course of the local contraction–expansion activity of the vein, as it fails to affect its electrical activity according to Kamiya and Nakajima (1955).

Discussion

In general, the present results well confirm the postulation that each contraction of veins in the plasmodial network squeezes the endoplasm forward, toward the frontal zone. This postulation was deduced earlier from the finding that all veins contract synchronously and their contraction phase coincides in time with the expansion phase of the frontal edge (Grębecki and Cieślawska 1978, Grębecki 1979 a, Cieślawska and Grębecki 1979 a and b, confirmed by Hejnowicz and Wohlfarth-Bottermann 1980).

An earlier attempt to verify this postulation (Grębecki and Moczoń 1978) gave generally positive results, but the obtained coefficient of correlation between the contraction phase and the forward streaming period was unexpectedly low. Its mean value was weakly significant ($\varphi = 0.16$). The present results are expressed by the much higher coefficient $\varphi = 0.35$, found in the principal veins crossing the central areas of the network (and even $\varphi = 0.42$ in more posterior zones). That proves a strong coincidence between the contraction of the vein and the flow of endoplasm toward the front.

This much stronger interdependence between the contraction and streaming phenomena could be probably demostrated in this study because of the improved experimental conditions. In the present experiments the whole surface of the investigated plasmodium was uniformly illuminated with a moderate light, whereas in the earlier ones the limited field of observation was exposed to a high light intensity and other parts of the plasmodium remained in shade. It is known that an intense light disturbs the synchronization of plasmodial veins (Takeuchi and Yoneda 1977). Moreover, the photophobic behaviour of plasmodium (Rakoczy 1973, Hato et al. 1976) allows us to suppose that it may be particularly sensitive to an uneven illumination of its surface.

The decrease of the coefficient of correlation between the contraction and streaming phenomena which has been observed in the anterior segments of the principal veins of plasmodium is certainly explained by the vicinity of the frontal zone, where the synchronous pattern of pulsation is replaced by the shifts of phase and wave phenomena (Baranowski 1978, Kołodziejczyk and Grębecki 1980).

The importance of the time relation between the pulsation rhythm and the shuttle streaming oscillations for the mechanism of locomotion of plasmodium is stressed by the fact that this correlation is much better pronounced when the contraction–relaxation cycles are more regular. The same meaning has the finding that activation of the frontal zone by shade which acts as a positive stimulus, distinctly improves the synchronization between the forward streaming periods and the contraction phases.

The discussed relation probably may be formulated in a more general manner: during contraction the endoplasm is squeezed from the decreasing body regions toward the increasing ones. Usually it corresponds to its transport from the network of veins toward the front. But when occasionally during several cycles a vein in the network is growing up, the endoplasm in it is flowing during contraction in the reverse direction.

Baranowski (1976) discussed the possible nature of the polarity factor which is responsible for the net gain in the endoplasm volume transported in the forward direction, in spite of the streaming oscillations. He suggested a possibility of such a phase shift between the contraction and streaming cycles which would ensure a larger vein's diameter for the forward streaming phase. This concept has been tested by Grębecki and Moczoń (1978) with negative results. It seems now that

the reasons of this failure were probably the same which led to the low correlation coefficient between the contraction and streaming obtained in the same study. Under present experimental conditions, when the whole plasmodium was evenly illuminated with moderate light and the thickest principal veins were used for investigation, it could be demostrated that in fact the forward streaming phase begins distinctly earlier than the contraction phase at the same control site (cf. Fig. 11). It means that in a maximally expanded vein the endoplasm usually flows forward, which confirms the original postulation of Baranowski (1976) but not our later objections (Grębecki and Moczoń 1978, Grębecki 1979 a).

It remains an open question how the forward streaming may begin earlier than contraction in the same central segment of a vein, if contraction is presumed to squeeze the endoplasm toward the front. Probably, the hydrostatic pressure in more posterior regions of plasmodium rises slightly earlier, than in the investigated middle part of a principal vein, but it remains yet a hypothetical explanation.

Although the vein's diameter factor may certainly contribute to the polarization of the volume transport in plasmodium, the time factor seems still to be at least of the same importance. The ratio 52 : 48 between the forward and backward streaming time was reported in the earlier study (Grębecki and Moczoń 1978). In the present experiments it was found to be even as high as 55 : 45 in the middle course of principal veins of the spontaneously migrating unaffected plasmodia. Hence, the forward streaming phase proves to be approximately 20% longer than the period of the reverse flow.

The importance of this relation for the polarization of the endoplasm transport and, therefore, for the locomotion of the whole plasmodium, is stressed by the fact that the application of a positive stimulus to the frontal zone (its activation by shade) leads to a further increase of the forward streaming time in detriment of the backward streaming phase. Their ratio is raised to 58 : 42. Shading of the front results also in an increased frequency of the contraction cycles. A similar increase in the pulsation frequency was earlier observed by Durham and Ridgway (1976) under the influence of positive chemical stimuli. On the other hand Anderson (1951) failed to demonstrate any change of frequency or of the forward streaming time in plasmodia which migrated toward the cathode in a DC field. It becomes interesting therefore to check whether the increase in the oscillation frequency, in the forward streaming time, and the improvement of the contraction–streaming synchronization, are generally related to the induction of any positive motor response of plasmodium, or are they dependent on the kind of stimulus.

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ACTA PROTOZOOLOGICA

VOL. 21 (No. 1) WARSZAWA, 1 XII 1982 pp. 55-59

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Contribution to Studies on the Role of External Cations in Excitability of Marine Ciliate *Fabrea salina*

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Synopsis. Fabrea salina was incubated in highly concentrated salt solution: 1.1 M NaCl + 0.1 M $MgCl_2$ + 0.016 M $CaCl_2$ + 0.005 M Tris/HCl (pH 7.2). Under these conditions the threshold concentration of KCl for induction of ciliary reversal (CR) was 132 ± 6.4 mM KCl. It could be lowered by decreasing concentration of external calcium and/or sodium whereas magnesium ions had no significant effect in this respect. Unlike in the case of *paramecium*, the duration of increased CR response in *Fabrea* did not depend on the ionic ratio: $[K^+] : [Ca^{2+}]^{1/2}$. At higher concentrations than 0.25 M KCl the induced CR could last longer than 48 h, persisting until the end of life of *Fabrea salina*.

Ciliates exposed to 0.01–0.02 M $BaCl_2$ (in presence of 0.016 M $CaCl_2$) showed frequently repeated short lasting CR responses ("Periodic Ciliary Reversal", Dryl 1961), which were associated with occurrence of depolarizing action potentials recorded by means of intracellular glass capillary microelectrodes technique. The achieved results brought evidence that marine ciliate *Fabrea salina* possess an excitable membrane like many fresh water ciliate protozoa.

Fabrea salina is a marine ciliate which lives in highly concentrated salt waters containing NaCl, $MgCl_2$, $CaCl_2$, KCl. Under natural conditions the general motile behaviour of *Fabrea* is similar to that known in fresh water ciliates, i. e., the animals show forward left-spiraling movement at relatively high swimming rate, approx. 1000 μm (per s). However, the ciliates react with longer or shorter lasting ciliary reversal (CR) when exposed to external stimulus of appropriate strength. CR response is expressed by left-helical backward swimming.

Since *Fabrea* is characterized by typical rounded shape with anteriorly located beak-shaped proboscis, it is easy to recognize the normal, forward movement or induced backward movement by applied external stimui.

The aim of the present study was to compare the behavioural response and the physiological properties of the cell membrane in fresh water and marine ciliates

Material and Methods

Fabrea salina strain (obtained from the Laboratory of Zoology, Ecole Normale Supérieure, Paris, France) was grown in medium: 1.1 M NaCl + 0.1 M $MgCl_2$ + 0.024 M $CaCl_2$ + 0.022 M KCl + 0.005 M Tris/HCl (pH 7.2) with addition of *Areoabcter aerogenes* suspension as a standard food.

Observations on behaviour were carried out in salt solutions of various composition and concentrations as indicated in the first four columns of Table 1. Before starting experimental procedure, the ciliates were incubated for 5–6 h in one from five solutions containing electrolites mentioned in columns 1, 2, 3, 4 (Table 1). KCl solution of desired concentration was prepared on the

Table 1

The behaviour of *Fabrea salina* at various levels of potassium ions in external medium of different ionic composition

Ionic composition of external medium*				Concentration thresholds of KCl for induction of CR (mM) (experimental data)	Calculated concentration thresholds of KCl for induction of CR according to $[K^+]$: $[Ca^{2+}]^{1/2}$ ratio (mM)
NaCl	Tris/HCl	$MgCl_2$	$CaCl_2$		
1	2	3	4	5	6
1.1 M	0.005 M	—	0.1 M	132±6.4	132
1.1 M	0.005 M	—	0.016 M	107±7.1	52.8
1.1 M	0.005 M	—	0.004 M	78±5.1	26.4
1.1 M	0.005 M	0.1 M	0.1 M	137±6.9	—
0.11 M	0.005 M	—	0.1 M	72±7.2	—

Concentration thresholds of KCl for induction of CR are expressed as arithmetical means (with standard deviations) from ten measurements. The criterion of threshold was the occurrence of short-lasting CR response in more than 50% of 25–50 tested ciliates.

* pH 7.2 was maintained in all applied solutions.

basis of above mentioned solutions. The criterion of threshold CR response towards KCl exposure was occurrence of first short-lasting CR response in more than 50% of 25–30 tested ciliates. Ten repeated measurements rendered possible to establish the threshold. The direct observations of behaviour were done under low magnification of optical microscope.

Electrophysiological studies on *Fabrea* were performed by means of glass capillary technique according to method applied by Kinosita et al. 1964.

Results and Discussion

It is well known from studies on *Paramecium* that duration of induced CR by potassium ions depends on the ratio of external cations: $[K^+]/[Ca^{2+}]^{1/2}$ = const. (Jahn 1962, Naitoh 1968). Consequently it was also found that the threshold concentration of potassium ions for induction of CR depends on the above mentioned

ionic ratio (Dryl and de Peyer 1970). According to recent views (Eckert and Naitoh 1972, Dryl and Jahn 1974) CR response appears as a result of competing action of K^+ on the cell-membrane bound calcium and consequent opening of hypothetical "calcium channels" (Eckert 1972) which is manifested by increase of level of calcium free ions in cilia and their reversed beat. The preliminary observations of the authors indicated that *Fabrea salina* responds also with CR to action of potassium and other CR-inducing agents. The first interesting difference found by the authors was that effects of higher concentrations of potassium (0.25 M KCl) on *Fabrea* were reflected by extremely long-lasting (more than 24 h) or even everlasting CR. Other behavioural differences of *Fabrea* are shown in Table 1. It is clear from data included in Table 1 that the established threshold concentrations for induction of CR responses do not depend on the ratio of external ions $[K^+] : [Ca^{2+}]^{1/2}$ since the experimental threshold values for 0.016 M and 0.004 M $CaCl_2$ (column 5) are much higher than it could be expected from the constance of Jahn's ratio (column 6). The presence of 0.1 M $MgCl_2$ in external medium did not influence the concentration threshold of KCl for induction of CR, while the tenfold decrease of NaCl caused marked decline of threshold (from 132 mN to 72 mM KCl). All these data suggest that calcium and sodium ions play an essential role in the excitability and motor response of *Fabrea*, although the ionic relations in the case of marine ciliate *Fabrea* seem to be much more complicated than in fresh water ciliates and at the present state of our knowledge is not so completely understood.

It should be pointed out that the experimental studies on *Fabrea* are limited because of very high sensitivity of this organism to decrease of NaCl and $CaCl_2$ concentrations. So far it was not possible to carry out experiments in medium of lower concentrations than 0.05 M NaCl and 0.001 M $CaCl_2$. At lower concentrations of above mentioned salts the ciliates were loosing proboscis and they perished within 15–20 min of exposure; under these conditions reliable observations on behaviour of *Fabrea* were not possible.

Parallel electrophysiological studies on *Fabrea* were performed in medium 1.1 M NaCl + 0.016 M $CaCl_2$ + 0.005 Tris/HCl (pH 7.2). In this medium the value of the membrane resting potential (RP) was 50–60 mV while the membrane resistance was 45–50 Mo as was recorded by means of glass capillary intracellular microelectrodes technique. Depolarization of RP was observed when the basic medium was enriched with potassium ions (0.12–0.30 M KCl), which induced long-lasting CR response. CR response disappeared and RP came back to initial value immediately after ciliates were transferred back to basic solution devoid of potassium ions.

In another series of experiments *Fabrea salina* exposed to 0.01–0.02 M $BaCl_2$ (in presence of 0.016 M $CaCl_2$) showed frequently repeated spontaneous depolarizing action potentials (AP) which were associated with short-lasting CR responses (so-called "Periodic Ciliary Reversal", Dryl 1961). Depolarizing AP-s were also observed after injection of intracellular inward current.

Typical AP-s induced by Ba/Ca factor are shown in the Fig. 1. Worthy to note is the rising phase of depolarizing spike with an overshoot to positive value, and falling phase of potential with marked phase of hyperpolarization, before reaching the level of RP. It is visible in Fig. 1 that the next AP appears after reaching "firing level" of depolarization.

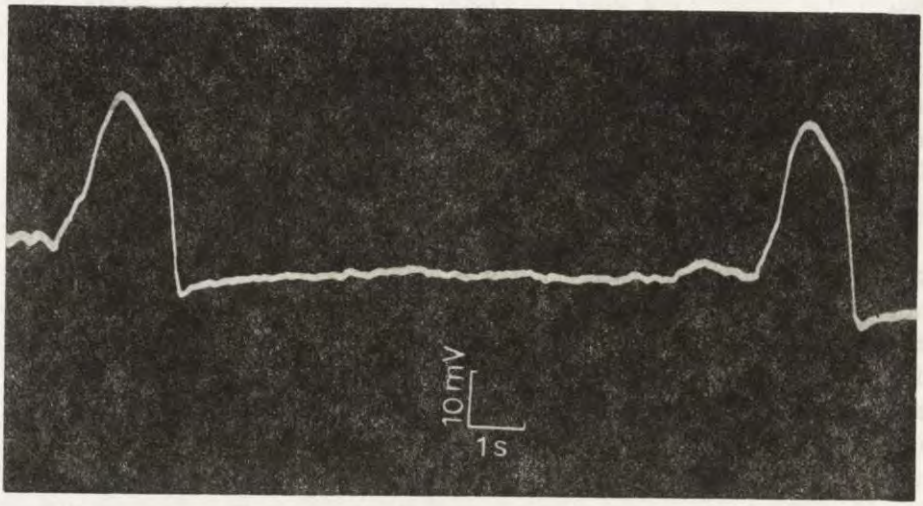

Fig. 1. Ba/Ca-induced depolarizing spikes associated with short-lasting ciliary reversal responses. *Fabrea salina* exposed to medium: 0.02 M $BaCl_2$ + 0.016 M $CaCl_2$ + 1.1 M NaCl + 0.005 M Tris/HCl (pH 7.2)

The recorded AP-s appear only in presence of sufficient concentrations of calcium ions and are of rather long duration (one or more seconds). This remainds us of similar findings reported for the first time by Kinosita et al. 1964 in the case of Ba/Ca induced PCR responses in *Paramecium*.

The achieved results in studies on behaviour and electrophysiology of *Fabrea salina* brought evidence that this marine ciliate possess an excitable cell membrane in agreement with the basic concept of Grundfest 1957.

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ACTA PROTOZOOLOGICA
VOL. 21 (No. 1) WARSZAWA, 1 XII 1982 pp. 61-67

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Modification of Motile Behaviour in *Paramecium octaurelia* by Cholesterol

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Synopsis. Experiments have shown a distinct influence of cholesterol on membrane excitability in *Paramecium aurelia*. The protozoans treated with cholesterol show a greatly prolongated duration of ciliary reversal, caused by an extracellular increase of K^+ concentration, as compared with the ciliary reversal time in control cells. No noticeable effect of cholesterol on the motor activity of the cilia in resting cells was observed. Treatment of *Paramecium* cells with labelled cholesterol also demonstrated that this substance penetrates readily into the cells in the course of incubation, thus confirming the supposition that the noted changes in membrane excitability in *Paramecium* may be due to the presence of cholesterol in the cell membrane. It is suggested that changes in ciliary activity in *Paramecium* cells under the action of cholesterol reflect the inhibitory effect of cholesterol on the efficiency of the active process in the cell, responsible for recovery of the activity of the cell ciliary system.

The presence of cholesterol ensures, on the one hand, mechanical stabilization of the cell membrane, and, on the other, markedly reduces the permeability of the biological membrane (Douticke et al. 1977), both in active and in passive transport, increases the microviscosity of this membrane (Feinstein et al. 1975) and affects the membrane potential (Shinozawa et al. 1979).

Disturbances in the physiology of the cell membrane caused by an increased cholesterol concentration in it as compared with phospholipid concentration were investigated in organisms in which cholesterol is present as a permanent component of the cell membrane. The problem seemed of interest whether a similar effect may be evoked by the presence of this lipid in *Paramecium octaurelia* cells in which cholesterol is not a permanent component.

Paramecium is an excellent model for investigating processes of cell membrane excitation and the ion exchange between the cell and the external environment connected with these processes. Membrane depolarization due (e. g., to a rise in K^+ ion concentration outside) occurs owing to the increased permeability to calcium, that is the flow of a calcium ions into the cell according to the concentration

gradient. Calcium ions flow through the calcium channels distributed in the membrane covering the cell cilia (Dunlap and Eckert 1976, Dunlap 1976, 1977, Eckert et al. 1976). The change of spatial orientation of ciliary beating in *Paramecium* occurs exactly at the moment when the electric reaction of the membrane starts (Machemer and Eckert 1973). Since the direction of ciliary beat directly reflects the intraciliary calcium concentration (Naitoh and Kaneko 1968), the K^+-induced duration of backward swimming (CR = ciliary reversal time), may be considered as an index of membrane excitability. The end of the reaction, the return of the membrane to the pre-excitation state, indicates a decrease of concentration and the attainment of the previous intraciliary calcium ion concentration. Ca^{++} extrusion occurs by way of active transport (Eckert 1972).

On the basis of the above presented data concerning ion exchange occurring during depolarization of the cell membrane in *Paramecium* and of the results of other studies (Drabikowski et al. 1972, Fisher and Moeller 1980, Kunishima and Izutsu 1970, Van den Berg and Betel 1971) investigations were performed on the changes in the physiology of the *Paramecium octaurelia* cell membrane under the influence of cholesterol. The results of preliminary experiments have been published earlier (Szydłowska 1980, 1981).

Material and Methods

Paramecium octaurelia (strain 299/S) was cultured according to the method of Soldo and Van Wagtendonk 1969) with the use of axenic medium designed by Soldo et al. (1966). To the standard culture medium (medium A) containing 5 μg/ml stigmasterol a cholesterol solution in ethanol was added. The final cholesterol concentration in the medium was 2 μg/ml (medium B), and the ethanol concentration did not exceed 0.2 %v/v. The third type of medium (medium C) contained a stigmasterol dose increased by 2 μg/ml, so that the final concentration of this sterol was 7 μg/ml. Cells from a 6-day culture were washed in 1 mM/l Tris-HCl buffer containing 1 mM $CaCl_2$, pH 7.3 (washing solution), taking advantage of the negative geotactic behaviour. This procedure was repeated three times. The washed and condensed cells were left in the buffer for 12 h. Three test solutions were prepared on the basis of the washing solution of the following KCl concentrations: 32, 48 and 64 mM. A drop of condensed ciliate suspension of about 5 μl volume was introduced into 2 ml of the test solutions of the above given KCl concentrations. The duration of the KCl-induced CR was measured with a stopper. The velocity of forward swimming of the protozoans was measured by the macrophotographic technique (Dryl 1958). Exposure time was 6 s.

Labelled (4-^{14} C) cholesterol (10 μC/l) was added to the standard medium (A) in order to determine the rate of cholesterol incorporation into the cells. The cells were three times washed with the washing solution by centrifugation at 800 g for 1 min. Thus prepared cells were treated with digitonin, a detergent with specific affinity to 3-betahydroxysterides. The following digitonin concentrations were applied with washing solution as base: 8×10^{-6} M, 20×10^{-6} M and 40×10^{-6} M. After 30 min of incubation in the detergent solution the cells were washed thrice by centrifugation. The labelled cholesterol was extracted from the digitonin solution and the washed cells with methylene chloride. The eluate was evaporated and the above mentioned amount of cholesterol in the particular fractions was added. The number of counts was determined with a Parker counter.

At the same time the survival of the cells was tested (Dryl and Mehr 1976) in order to establish whether the eluted cholesterol is derived from the cell membrane.

Results

As seen in Table 1 and Fig. 1, the duration of CR for the protozoans cultured in various types of medium differs markedly. The effect of cholesterol on the degree of excitatibility of the cell membrane in *Paramecium* is distinctly noticeable in the prolonged duration of CR induced by the change in the extracellular potassium

Table 1

Dependence of duration of K^+-induced ciliary reversal of *Paramecium* on culture medium (A, B, C) at three K^+ concentrations

Medium	K^+-induced backward swimming (in s)		
	32 mM KCl	48 mM KCl	64 mM KCl
A	26±2.01	35±4.38	45±1.73
B	32±2.26	46±5.77	54±3.53
C	29±3.34	43±2.61	50±2.66

ion concentration. For the control cells the CR time was 26 s, whereas for medium with cholesterol added (B) it was about 39 s. Similar, though less pronounced, changes in excitability of the protozoans were produced by increasing the stigmasterol concentration in the culture medium (C) (Fig. 1).

Analysis of the tracks of the animals swimming forward did not demonstrate a significant influence of cholesterol and the increased stigmasterol dose on this type of movement. Both the swimming velocity and other parameters of the recorded track remained unchanged for all types of cells (culture media A, B, C).

In other experiments advantage was taken of the property of digitonin of forming a stable complex with sterols, this steride detergent was used as "index" of labelled cholesterol in the cell membrane. Measurements of labelled cholesterol

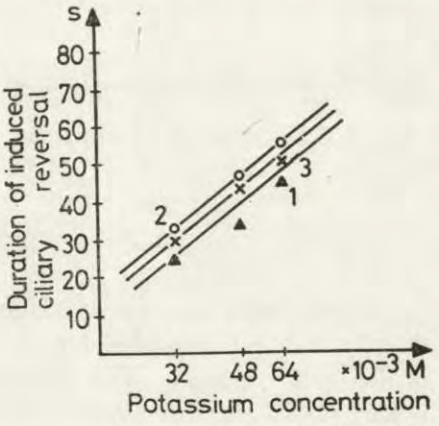

Fig. 1. Duration of K^+-induced backward swimming of *Paramecium* at three different K^+ concentrations in external test solutions. *1* — control cells (medium A), *2* — cholesterol-treated cells (medium B), *3* — cells treated with higher concentration of stigmastero (medium C)

in the incubation medium with low digitonin concentration (8×10^{-6} M) indicate that this cholesterol is derived from the outer side of the cell membrane (Table 2). The above mentioned digitonin concentration did not cause in the course of the experiment cell lysis, the survival of *Paramecium octaurelia* in these experiments was close to 100% (Table 3 and Fig. 2).

Table 2

Relation between relative amount of cholesterol within the cells after digitonin treatment and the amount of cholesterol bound to digitonin in the washing medium. The incubation time was 30 min

Digitonin concentration	Ratio of membrane-bound cholesterol to the cholesterol-digitonin complex in the washing medium
8×10^{-6} M	6.4
20×10^{-6} M	1.9
40×10^{-6} M	0.9

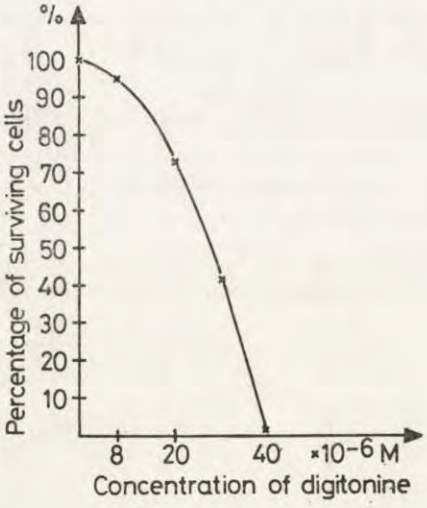

Table 3

Percentage of surviving cells at different concentrations of digitonin in the washing solution. Incubation time was 30 min

Digitonin concentration	Percentage of surviving cells
8×10^{-6} M	96
20×10^{-6} M	73
40×10^{-6} M	0

Fig. 2. Percentage of cells surviving in different concentrations of digitonin

Discussion

The results of experiments concerning the influence of the cholesterol level on the physiological state of biological membranes suggest that this compound is one of the most important lipid components of most biological membranes and that it may play a significant role in the regulation of membrane permeability. A rise

or fall in the cholesterol level in the membrane modifies the permeability of mammalian erythrocyte membranes to small non-electrolyte particles and anions (Douticke et al. 1977, Grunze and Deuticke 1974). Modulation of the membrane permeability to cations by cholesterol causes wide changes in processes of passive and active transport. The enrichment in cholesterol or its removal from the membranes of human red blood cells affects Na^+–K^+ cotransport (Wiley and Cooper 1975). The sitauation is similar as regards phosphatidylcholine-rich mammalian red cell membranes where leak and active Na^+–K^+ transport are greatly dependent on the membrane cholesterol (Poznansky et al. 1973, Kroes and Ostwald 1971, Cooper et al. 1975). Cholesterol adsorption on the membrane vesicles of the endoplasmic reticulum from rabbit leg and back skeletal muscle cells depresses both the Ca^{++}-dependent ATPase activity and Ca^{++} uptake simultaneously with wide morphological changes in the ultrastructure of these vesicles (Drabikowski et al. 1972).

The electrophysiological and behavioural investigations of Naitoh et al. (Kinosita and Murakami 1967, Naitoh et al. 1972, Naitoh and Kaneko 1973), concerning the problem of regulation of the movements of *Paramecium* as well as biochemical studies of the basis of membrane excitability in this protozoan indicated that the cell normally maintains a low intracellular (cytoplasmic) level of Ca^{++} by an active Ca^{++} extrusion pump. Electric or chemical stimulation produces electric depolarization of the cell membrane and opening of the membrane channels through which Ca^{++} flows into the cytoplasm according to the electrochemical gradient. The increase of cytoplasmic Ca^{++} triggers CR which lasts until the Ca^{++} pump reduces the concentration to the value prevailing before stlmuiation (Andrivon 1974, Doughty 1978, Eckert 1972, Machemer 1974 a). It results from investigations of Browning and Nelson (1976) that a depression of environment temperature of the cells influences the rate of calcium outflow from them. This is characteristic for active transport systems, whereas inflow of calcium is a passive process dependent on an electric or chemical mechanism opening the calcium channels in the *Paramecium* membrane. It is known, on the other hand, that depression of temperature causes a significant protraction of CR duration (Hildebrand 1978). These observations confirm the behaviour model of the membrane in excited and nonexcited state.

The results of excitability measurement shown in Table 3 indicate that cholesterol as well as artificially introduced stigmasterol into the *Paramecium* cell membrane modifies greatly the behaviour of these cells. The presence of this lipid in the membrane prolongs the time of backward swimming of the cells under the influence of a chemical stimulus (KCl), without producing major changes in the behaviour of resting cells. It may be concluded, on the basis of the presented experimental observations on the influence of cholesterol on the behaviour of *Paramecium* cells and its role in modifying numerous vital functions in other non-protozoan cells that an analogous effect of calcium pump inhibition and prolongation of the dur-

ation of the state of CR, as in the case of depressed temperature, seem quite probable. The above facts would, therefore, suggest that the influence of this lipid on the functional state of biological membranes must be almost universal, notwithstanding whether this substance is a natural component of the membrane or is introduced experimentally.

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Studies on the Lectin Binding Capacity of the *Tetrahymena*

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Synopsis. Binding of fluorescein labelled lectins to the membrane of *Tetrahymena* was studied using a quantitative cytofluorimetric method. Various lectins were able to bind to the membrane of *Tetrahymena*. Examinations with seven different lectins binding to known groups showed that lectins interacting with simple sugars as their ligands are more readily bound than those requiring the presence of oligomeres for binding.

The *Tetrahymena*, although by nature devoid of a hormonal regulation, does possess membrane structures capable of binding hormones, including those of vertebrates (Csaba and Lantos 1973, 1975 a and b, Csaba et al. 1977, 1979). However, the receptor-like structures of the unicellulars necessarily differ to a certain degree from those of the vertebrates. Since they differentiate the hormones from their precursors by preference of the latter (Csaba and Németh 1980). This suggests that like hormones, the receptors, too, undergo an evolutionary process (Barrington 1978, Csaba 1980).

It was shown experimentally (Csaba and Lantos 1977) that longer lasting treatment of *Tetrahymena* with a hormone results in the amplification of certain binding sites, either through a quantitative increase of sites already existing, or by arising of new membrane structures which were lacking in the intact (non-exposed) cell. Whatever the underlying mechanism, it involves in all probability an alteration of the cell membrane which also applies to the membrane's mono- and oligosaccharide components. With the saccharide components of the intact membrane known, the changes interpreted as specific response can be easily followed up by binding of FITC- (Fluorescein Isothiocyanate)-labelled lectins. In view of this we examined the lectin-binding capacity of the *Tetrahymena* for first approach.

It was demonstrated earlier (Bleyman et al. 1975, Frisch et al. 1976, Wise 1975) that unicellular organisms are able to bind certain lectins. Our own scanning

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electron microscopic studies (Csaba and Madarász 1979) revealed that concanavalin-A (con-A) binds selectively to given details of the membrane, above all to cilia of the cell body at their tips, as preferential sites. In the present study seven lectins with known binding affinity were examined for binding to *Tetrahymena* by fluorometric analysis.

Material and Methods

Tetrahymena pyriformis cells (strain GL) were fixed in 4% neutral formalin (pH 7.0 in 0.1 M phosphate buffer) for 5 min, and were washed in two changes of phosphate buffered saline (PBS). Seven experimental groups were formed, one for each lectin. The volume of each group was 600 μl. The appropriate FITC-labelled lectin was added to each cell suspension. The protein concentration was 400 μg/ml with each lectin. The preparations were incubated for 1 h at room temperature, washed in two changes of PBS, transferred to a slide, and air-dried.

The lectins used were either prepared in this laboratory, or were commercial preparations (Table 1).

The *Tetrahymena* cells were examined for the intensity of fluorescence in a Zeiss Fluoval cytofluorimeter, by scanning technique. Fluorescence was recorded with a Zeiss K 100 linear compensograph, and its numerical value was calculated from the integrals of the areas beneath the curve.

Conjugation of the lectins with FITC (Isomer 1., BDH Chemicals Ltd. Poole, England) was carried out as described by Loor (1974).

The specificity of binding to *Tetrahymena* was checked with two lectins of similar ligand affinity, Concanavalin A (con-A) and *Lens culinaris* (lentil) lectin, as follows:

(1) It was examined whether pretreatment with con-A would depress the binding of later superposed lentil lectin. *Tetrahymena* cells were, after fixation and washing in PBS, incubated in the presence of con-A (400 μg/ml) for 1 h at room temperature, washed twice in PBS, and incubated again for 1 h at room temperature with FITC-labelled lentil lectin also added at 400 μg/ml concentration. This was followed by two washings in PBS, transfer to a slide, and drying. Cells exposed to FITC-labelled lentil lectin alone served as control.

(2) Superposition of α-D-glucose and α-D-mannose on FITC-labelled con-A and lentil lectins.

Fixed, washed *Tetrahymena* cells were incubated in the presence of FITC-labelled con-A or lentil lectin (each added at 400 μg/ml) for 1 h at room temperature, washed in two changes of PBS, exposed to 20 mM glucose or mannose for 20 min at room temperature, washed again twice in PBS, transferred to slides, and dried.

The reported data are based on three experiments.

Results

The degree of binding of the lectins tested is shown in Fig. 1. All lectins were bound in a minor to major degree. In the falling sequence, the lectins of *Pisum sativum* (pea), *Lens culinaris* (lentil) and *Canavalia ensiformis* (con-A) were abundantly bound, while those of *Helix pomatia* (common snail), *Datura stramonium* and *Phaseolus vulgaris* (bean: phytohaemagglutinin) were bound in a lesser degree.

Table 1

Characterization of the lectins used in the experiments

Origin or name of lectin	*Pisum sativum* (pea)	*Canavalia ensiformis* (con-A)	*Lens culinaris* (lentil)	*Helix pomatia* (common snail)	*Glycine max* (soya bean)	*Phaseolus vulgaris* type S (garden bean)	*Datura stramonium* (datura)
Ligand*	D-glucose; D-mannose; Methyl-α-D--mannoside; N-acetyl-D--glucosamine	Glucose and its derivatives; Mannose and its derivatives; Fructo-furanosides	Glucose and its derivatives; Mannose and its derivatives, N-acetyl-D-glucosamine	N-acetyl-D-galactose-amine, N-acetyl-α-D-galactose--amines, N-acetyl--α-D-glucose-aminides	N-acetyl-α-D--galactose-amines, and α-D--galactopyranosides	N-acetyl-galactose-amine, its derivatives and oligomeres	Oligomeres of N-acetyl-glucosamine
FITC/protein ratio in the solution used	2.02	0.28	1.22	1.17	3.14	1.7	0.74
Molecular weight	107 000	110 000	49 000	79 000±4000	120 000	128 000	85 000
Molarity at 400 μg/ml	3.73×10^{-6}	3.63×10^{-6}	8.16×10^{-6}	5.6×10^{-6}	3.3×10^{-6}	3.12×10^{-6}	4.7×10^{-6}
Origin or producer (reference)	Entlicher et al. (1970)	SERVA (Heidelberg)	Howard et al. (1971)	Hammarström and Kabat (1969)	Vretblad (1976)	SERVA (Heidelberg)	Kilpatrick and Yeoman (1978)

* Listed in the sequence of ligand affinity.

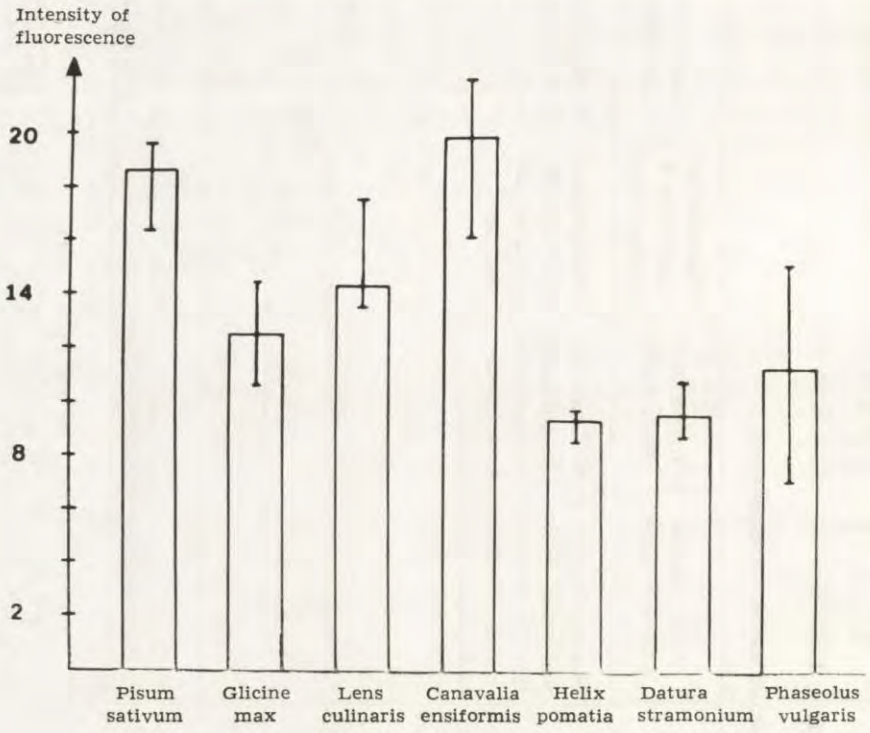

Fig. 1. Lectin binding of *Tetrahymena pyriformis* GL cells

Examinations for the specificity of binding showed that on combined treatment with con-A and lentil lectin, noted for a similar ligand (receptor) affinity, pretreatment with con-A depressed the binding of superposed lentil lectin considerably (51.73%), in a statistically highly significant degree (Fig. 2). α-D-glucose or α-D-mannose superseded con-A and lentil lectin (Fig. 3). The differences were significant ($p < 0.05$ and 0.01 respectively).

Discussion

The fact that all lectins were able to bind to the *Tetrahymena* indicates that the membrane of the latter does carry the structures — receptors, ligands — required for the specific binding of lectins. However, variations in the degree of binding between lectins have suggested a dissimilar frequency of the structures serving as binding sites. The lectins abundantly bound were those known to bind to simple sugars, while the ones bound in a lesser degree were those with exclusive affinity to amino sugars or oligomeres (Monsigny et al. 1979). It follows that the terminal saccharibes of the membrane glycoproteins are non-repeatable saccharide elements.

and most of them are probably not amino sugar like in nature. In the case of the *Pisum sativum* and *Lens culinaris* lectins, most abundantly bound of all, affinity was lowest for N-acetyl-D-glucosamine, which suggests a lesser binding also of the soya lectin, known to interact with amino sugars.

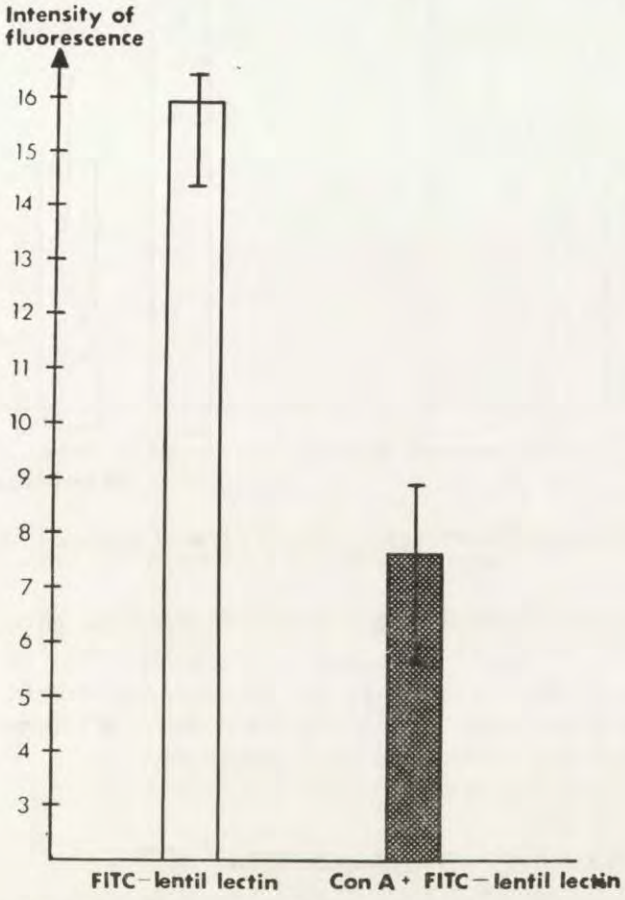

Fig. 2. Effect of pretreatment with con-A on binding of FITC-labelled *Lens culinaris* lectin to *Tetrahymena*

Evidence of the specificity of lectin binding was obtained from pretreatment and superposition experiments. As shown in Table 1, the binding of con-A and lentil lectin is associated with the same chemical groups. *Tetrahymena* cells pretreated with con-A bound half the quantity of superposed lectin compared to those not pretreated. Also, α-D-mannose and α-D-glucose superseded a considerable part of bound con-A and *Lens culinaris* lectin, respectively, on the Tetrahymena cells.

The superseded quantitatives of con-A and lentil lectin were identical. It is known that the membrane glycoproteins of mammalian cells do not normally contain glucose (Wright 1979).

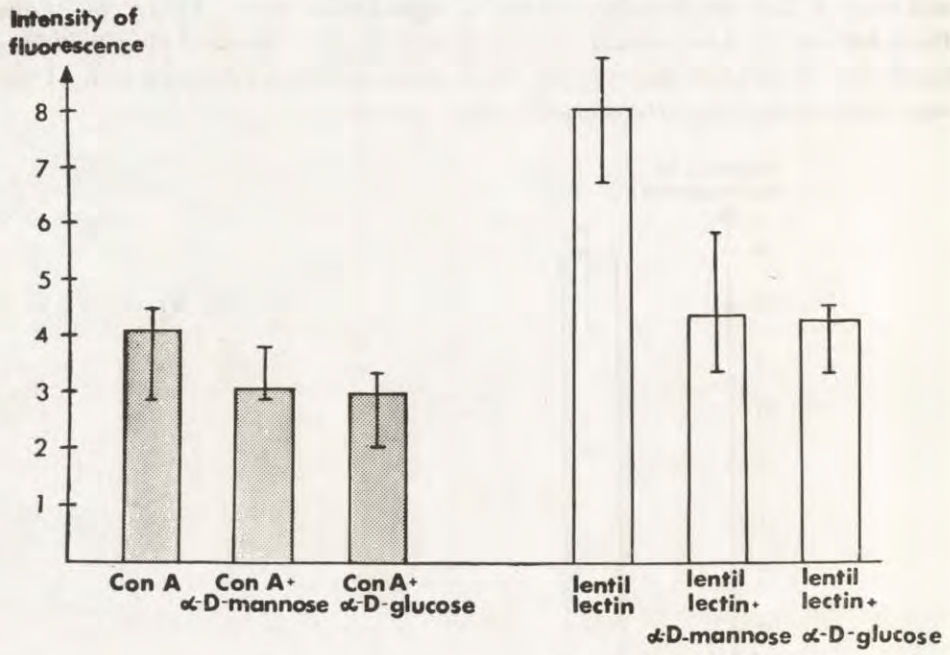

Fig. 3. α-D-glucose and α-D-mannose supersede FITC-labelled con-A and *Lens culinaris* lectin bound to receptors of the *Tetrahymena*

In view of this, one might postulate that the membrane glycoproteins of the *Tetrahymena* are also devoid of terminal glucose groups. Since the two lectins in questions can equally bind to glucose and mannose, each may be superseded by either in a membrane-bound state. The identical degree of superposition suggests that both lectins bind to the same sugar component.

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ACTA PROTOZOOLOGICA

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Effect of Lasting Treatment with Histamine Antagonists on Lectin Binding of *Tetrahymena*

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Synopsis. Culturing of *Tetrahymena* cells in the presence of histamine for 4 days altered the lectin binding potential of the membrane. Presence of histamine antagonists in similar condition altered lectin binding differently from histamine, indicating that the cell membrane is capable of differentiating histamine from its antagonists. The H_2-antagonist methiamide, which is structurally closest related to histamine, was most similar to it also in respect of influence on lectin binding. Long-term (4-day) cultivation in absence of histamine or other active substance did itself induce membrane alterations, to judge from the increase of datura-lectin binding with progressing time.

The unicellular *Tetrahymena* possesses membrane binding sites which are capable of interacting with hormones of higher animals (vertebrates), and thereby display a receptor-like function (Csaba and Lantos 1973, 1975, 1977, Csaba 1980). Since the receptors usually consist of oligosaccharide chains which bind to proteins, it is not surprising that certain lectins, which recognize oligosaccharides specifically and bind to them, can also bind to the *Tetrahymena* (Csaba and Madarász 1979, Frisch et al. 1976, Kovács and Csaba 1981). It follows that as established also by Williams et al. (1980), the *Tetrahymena* represents a useful model for experimental study of the eukaryote cell membrane. This is still more feasible, if it is taken into consideration that overlaps occur on binding sites of the *Tetrahymena* in the same manner as in mammalian cells. Our earlier studies along this line (Kovács and Csaba 1981) have shown that insulin and histamine, known to overlap concanavalin-A (con-A) on the membrane binding site of mammals (Sandra et al. 1979) also overlap it on the receptor of the *Tetrahymena*. It follows that histamine

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inhibits the binding of con-A, and vice versa. In the present experiment *Tetrahymena* cells durably exposed to histamine antagonists were examined for lectin binding, to obtain information whether or not structurally similar or dissimilar antagonists induce the same membrane alterations as histamine or, in other words, whether or not the cell membrane can differentiate histamine from its antagonists.

Material and Methods

We used the histamine antagonists (N,N-dimethyl-N'-(p-chlorobenzoyl)-N'-(2-pyridil)-ethylene-diamine] HCl [Suprastin, EGYT, Budapest), 2-methyl-9-phenyltetrahydropyridine bitartarate (phenindamine, Pernovin, Chinoin, Budapest), N-methyl-N'-[(5-methyl–imidazol-4-yl) methylthio] ethyl (thiourea) methiamide, Smith, Kline and French, U. K.), and a commercial histamine preparation (Reanal, Budapest), for treatment of *Tetrahymena pyriformis* GL cells cultured in Bacto trypton medium (Difco, Michigan) at 28°C.

Culturing in the presence of antagonist, histamine, or no active substance (control series) for 4 days was followed by washing and 1 h rest in Losina-Losinsky solution, fixation in 4% neutral formaline, and two washes in phosphate buffered saline (PBS). Part of the cells so treated were additionally exposed to 10^{-6} M histamine for 5 min, followed by three washes in PBS. FITC (Fluorescein Isothiocyanate) labelled lectins were finally added to each series and the intensity of fluorescence was determined by a Zeiss cytofluorimeter and are given as arbitary units in the Figures.

The lectins used were derived from the following sources: pea (*Pisum sativum*) (after Entlicher et al. 1970), Concanavalin A (con-A, *Canavalia ensiformis*, Serva, Heidelberg), lentil (*Lens culinaris*) (after Howard et al. 1971), *Helix pomatia* (after Hammarström and Kabat 1969), Soya bean (*Glycine max*) (after Vretblad 1976), garden bean (*Phaseolus vulgaris*, Serva, Heidelberg), *Datura stramonium* (after Kilpatrick and Yeoman 1978). Each lectin was applied at 400 μg/ml concentration. Significance was evaluated by Student's *t*-test.

Results and Discussion

The experimental results are shown in Figs. 1 and 2. Figure 1 shows that the antagonists altered the membrane pattern differently from histamine: while 4-day exposure to histamine equally enhanced the binding of lens-lectin and con-A, the antagonists enhanced it hardly and in a lesser degree, respectively. The effect of the antagonists tested was practically uniform, showing no variation with the structural differences. The cells re-exposed to histamine, then to lectin after 4-day primary exposure to histamine and a subsequent wash, showed differences in the binding of lens-lectin and con-A, known to bind to similar sugar components. While histamine depressed the binding of con-A similar to the acute experiment (Kovács and Csaba 1981), it failed to depress the binding of lens-lectin. It should be noted that in this condition the curve for methiamide, the H_2 antagonist most closely related to histamine, bore the closest resemblance to the histamine curve.

The control cells cultured for 4 days showed a considerable increase of datura-lectin binding over those cultured only for 2 days (Kovács et al. 1981). In the light of other experimental observations (Kovács and Csaba 1981) it seems highly probable that the binding of lectins carrying dimeric and oligomeric ligand tends to increase with the ageing of the cell population. This suggests that the age-related reassembly of the cell membrane is due to a membrane destruction consequent upon insufficient nutrition due to increase of the population. It has been reported

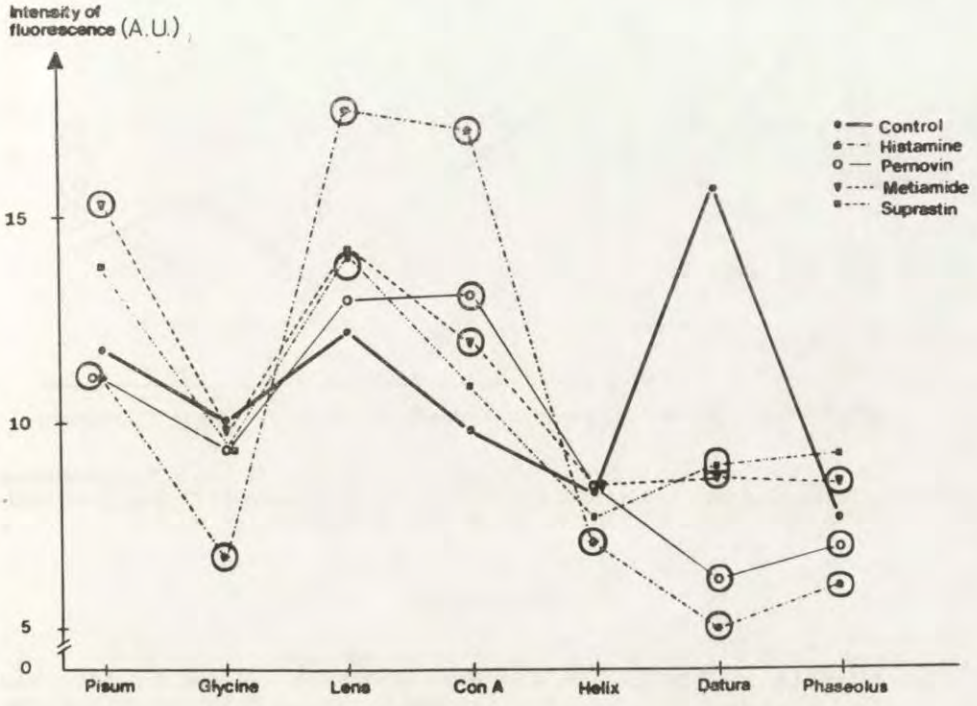

Fig. 1. Effect of treatment with histamine or its antagonists on the lectin binding capacity of the *Tetrahymena*. The points marked with a circle are significant ($p < 0.01$ or < 0.05) relative to control

(Gebbinck 1980) that trypsinization also alters the conditions of lectin binding, since digestion of external membrane proteins by the enzyme "exposes" additional saccharide components to which lectins can bind. In the given system the cells might compensate the diminution of nutriment resources by self-degradation or, since nutriments also serve as impulses for receptors (Lenhoff 1974), by a quantitative decrease of receptor sites. This kind of change in the reception of datura-lectin can be compensated by addition of histamine or of its antagonists.

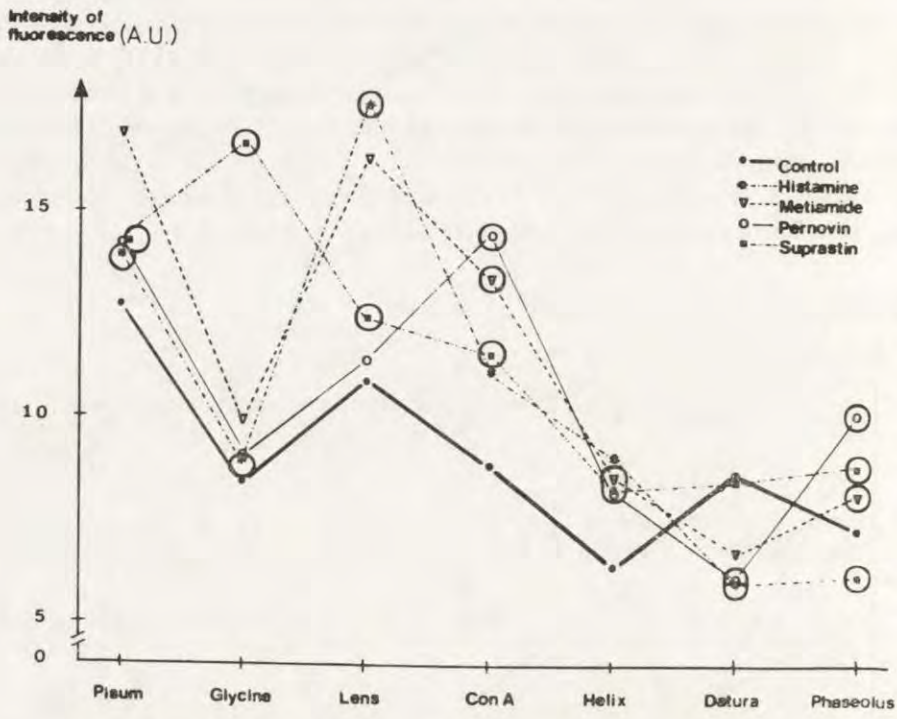

Fig. 2. Effect of treatment with histamine and its antagonists on lectin binding to the *Tetrahymena* following 5-min re-exposure to histamine after fixation. The points marked with a circle are significant ($p < 0.01$ or < 0.05) relative to control

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ACTA PROTOZOLOOGICA

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The Radioprotective Effect of Some Scavengers of the OH Radical on the Radiation-Induced Changes in the Sensitivity of the *Protozoa* Membranes to Sodium Chloride Concentration

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Synopsis. The *Spirostomum ambiguum* protozoa were exposed to 0.1 Mrad gamma radiation in the presence of some $\dot{OH}$-radical scavengers. After irradiation the protozoa were placed in the 30.5 mM sodium chloride solution and the surviving percentage after certain storage time was estimated. The linear relation between the reaction rate k of $\dot{OH}$ radical scavengers and their radioprotective effect on the protozoa was found.

The gamma radiation acts on protozoa living in water mainly via indirect mechanisms. The cells of such protozoa contain ca. 90% of water (Grobnicka and Wasilewska 1925). The energy of ionizing radiation is absorbed by the cell components according to their share in total mass of the cell. The highly reactive water radicals (i. e., $\dot{OH}$, $\dot{H}$ and e_{aq}) take part in indirect action of these ionizing radiation, causing radiation damage in biological structures. The significant effect on this indirect action can be assessed by observing the reduction in the radiation effect due to the introducing radical scavengers. Balcerzak (1973) demonstrated that in lethal dose range it is the $\dot{OH}$ radical which is mainly responsible for the mortality among the protozoa.

Sassaki and Matsubara (1977) reported that the gamma radiation-induced aberration of chromosomes in human lymphocytes can be mainly attributed to the action of $\dot{OH}$ radicals. The ionizing radiation in the sublethal dose range causes an outflow of potassium ions from the interior of the cell (Shapiro and Kollmann 1968, Myers and Johanson 1974, Rink 1975, Chapman and Sturrock 1975, Balcerzak 1978) and facilitates the transport of sodium ions into the cell (Shapiro and Kollmann 1968, Rink 1975). Therefore the ionic equilibrium between the interior of the cell and the environment is perturbed. It also leads to a reduction of the energy barrier for the transport of phosphate ions across the protozoa membrane (Balcerzak 1979). The ionizing radiation in the sublethal dose

range increases the sensitivity of protozoa cell membranes, which depends on the concentration of sodium chloride (Balcerzak and Rostkowska 1978). The scavenger method offers an occasion to gain information about the primary processes related to the generation of damage in cell membranes. In this work we have studied the effect of some OH scavengers on the radiation-induced change in the sensitivity of protozoa cell membranes to an exposure of the 30.5 mM sodium chloride concentration. In particular, the dependence of the scavenging rate of OH radical on radioprotective effect of these substances is investigated.

Materials and Methods

Culture of *Spirostomum ambiguum* were grown at 20° C in 1 l glass flasks on the nutrient of the following compositions: KCl 0.05 mM, NaCl 2.0 mM, $MgCl_2$ 0.2 mM, $CaCl_2$ 0.5 mM, KOH 0.01 mM, Na_2HPO_4 0.05 mM, NaH_2PO_4 0.05 mM in 1 l distilled water. Some boiled oat grains were introduced into the culture as a source of food for bacteria on which ciliate was fed. pH was kept to be 7.0.

The protozoa designed for studies were collected from the culture and rinsed with 0.1 mM phosphate buffer. Eight bacteriological test tubes with 30.5 mM NaCl solution were prepared and 100 animals were introduced into each of these tubes. After certain time the number of living organisms was determined. The percentage of the surviving animals was estimated after one hour periods. For the estimation of the surviving animals the lysoamine pigment was used. In another experiment eight bacteriological test tubes each containing 100 animals with 0.1 mM phosphate buffer were exposed to 0.1 Mrad gamma radiation with dose rate 0.1 Mrad/h. The samples were irradiated in the Institute of Applied Radiation Chemistry of Łódź Technical University.

The protozoa were after irradiation transferred to 30.5 mM NaCl solution and the percentage survival of animals was estimated. The last series of experiments include the use of OH scavengers. The particular scavengers with the 0.1 mM concentration were separately introduced to respective sets of the test tubes with protozoa 20 min before their exposure to gamma radiation.

After the irradiation the animals were exposed to 30.5 mM NaCl solution and the percentage of survivals was determined. All the experiments were carried out at the constant temperature equal to 20° C.

Results and Discussion

The proportional survival of the unirradiated protozoa as well as the protozoa irradiated with 0.1 Mrad dose and acted by the 30.5 mM NaCl solution is presented in Fig. 1. The 50% survival time of the irradiated animals was reduced to (1.5 ± 0.1) h as compared to (22 ± 0.3) h found for the protozoa processed without irradiation.

Figure 2 presents the protective effect of several OH scavengers introduced into the medium containing protozoa 20 min before the protozoa were exposed to gamma radiation and then placed in the 30.5 mM NaCl solution. The presence of the OH scavengers in the medium containing protozoa during the irradiation leads to a prolongation of the 50% survival time in comparison with the protozoa irradiated

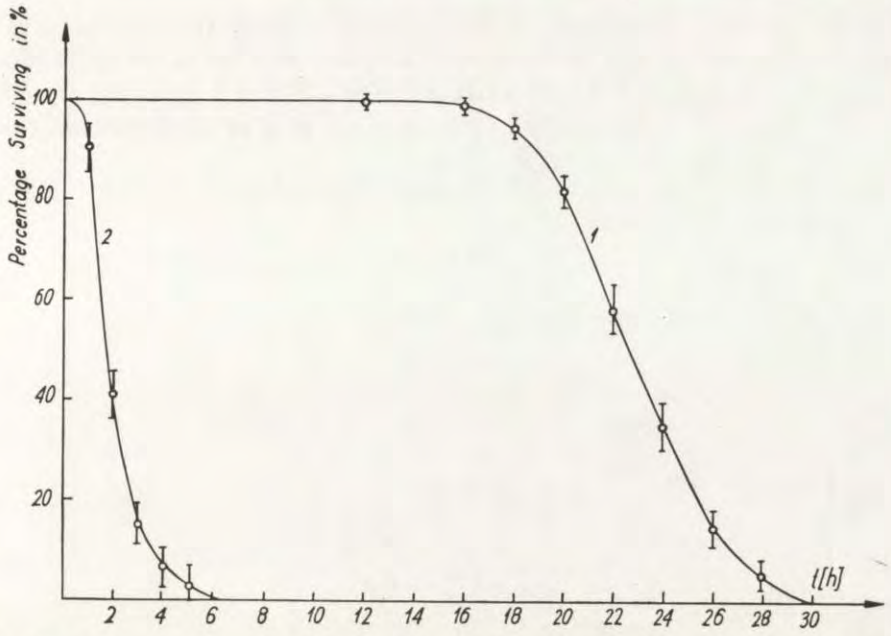

Fig. 1. Sensitivity of irradiated and unirradiated *Spirostomum ambiguum* to NaCl at concentration 30.5 mM. Ordinate: the percentage of surviving animals. Abscissa: the time of exposure to 30.5 mM NaCl. 1 — unirradiated animals, 2 — animals irradiated with 0.1 Mrad dose

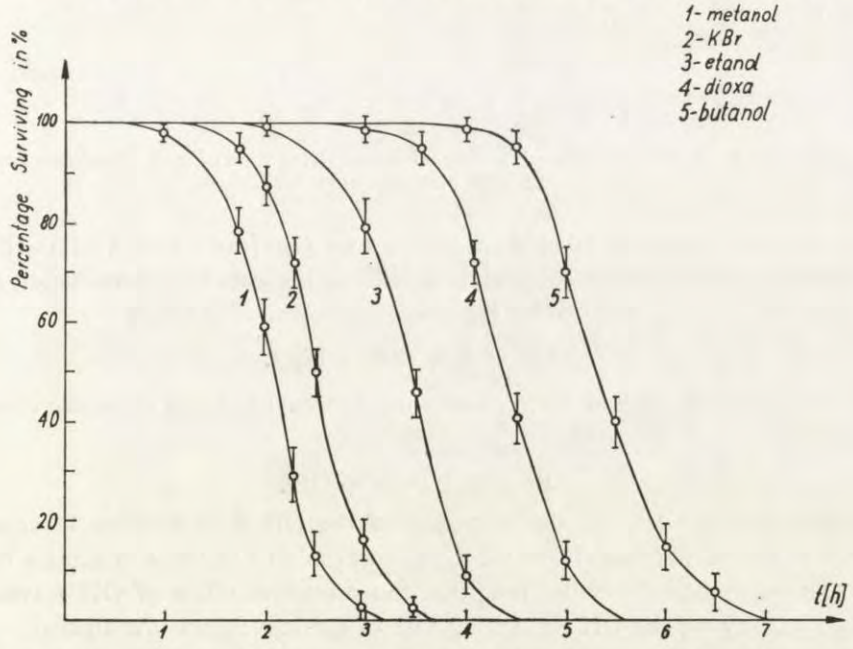

Fig. 2. Protective effect the $\dot{O}H$ scavengers with the concentration 10^{-4} M on the sensitivit of *Spirostomum ambiguum* irradiated with 0.1 Mrad dose. Ordinate: the percentage of surviving animals. Abscissa: the time of exposure to 30.5 mM NaCl. *1* — methanol, *2* — potassium bromide, *3* — ethanol, *4* — dioxane, *5* — butanol

with the same dose but without radioprotective substances. The survival curve presented in Fig. 2 allowed us to estimate the 50% survival time of the animals ($t_{s\text{-}50}$) irradiated in the presence of several OH scavengers. Figure 3 shows the dependence of reaction rate of scavengers with radicals on the $t_{s\text{-}50}$ of the irradiated animals placed in 30.5 mM NaCl solution.

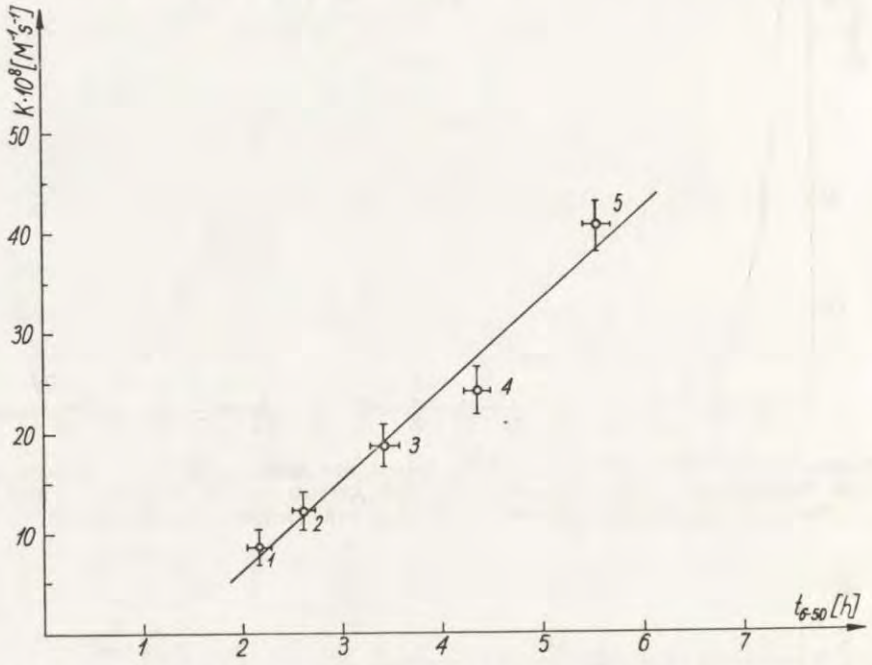

Fig. 3. Dependence of the $\dot{OH}$ scavenging rates on the surviving time of 50% of animals acted by 30.5 mM NaCl solution

The reaction rates were taken from the data by Dorfman and Adams (1973). The chemical reaction of hydroxyl radicals with such substances as methanol, ethanol, butanol, dioxane proceed by reduction hydrogen abstraction

(1) $$\dot{OH} + RH \rightleftarrows \dot{R} + H_2O.$$

In the case of the use of bromide ions the hydroxyl radicals cause the electron transfer

(2) $$Br^- + \dot{OH} \rightleftarrows Br + OH^-.$$

These reactions lead to the reduction of the OH concentration in aqueous medium in the membranes and in the interior of the cells exposed to gamma radiation. Our experimental results show that the protective effect of OH scavengers depends linearly on the OH scavenging rate of the scavengers investigated.

On the basis of these findings it can be assumed that the radiation-induced damage of ciliate cell membranes, leading to a change in the sensitivity to sodium

chloride concentration, is due to the action of OH radicals. In the range of both the lethal and sublethal doses the hydroxyl radicals cause damage in cell membranes and cell organs. It seems that this damage can lead to the death of the cell. It should be emphasized that it was possible to obtain those results owing to the particular choice of an object for the experiment. This object contains large amount of water and lives in aqueous medium. In this case the indicret action of radiation is especially evident.

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Spectrophotometric Identification of a Carotenoid Pigment in the Resting Cysts of a Hypotrichous Ciliate, *Laurentiella acuminata*

Received on 10 August 1981

Synopsis. The pigmentation of the resting cysts of the hypotrichous ciliate *Laurentiella acuminata* has been studied by microspectrophotometry. Visible absorption spectrum of individual cysts *in vivo* showed a great similarity to that of a carotenoid, possibly an α-carotene, isolated from them. This similarity suggests that the carotene is responsible for cysts pigmentation. The pigmentation increased when vitamin A was added to the cultures. A similar carotene was found in algae fed to the ciliate. It seems that an active accumulation of algal carotene occurs during cystic pigmentation development.

The resting cyst stage as well as the last precystic phases of the encystment–excystment cycle of some hypotrichous ciliates (Matsusaka 1976, 1979, Jareño 1977) have a characteristic pigmentation, which is absent during the biological cycle of these cells. Nevertheless, the nature, origin and possible function of this pigmentation is as yet unknown.

In cultures of these ciliates, encystment occurs when food becomes a limitant factor. During the encystment, structural and pigmentary changes have been observed (Gutierrez et al. 1979). The following six stages were chosen on the basis of observations on the cell pigmentatton during its encystment: Stage V (vegetative cell), stage A (precystic semitransparent), stage B (precystic transparent), stage C (precystic pigmented), stage D (spherical shape without cyst wall) and stage E (resting cyst). The yellowish colour (stages C, D and E) persists in the resting cyst as long as this dormancy stage lasts, dissappearing in the excysted cell.

The purpose of this communication is to further investigate the origin, function and ecological significance of resting cyst pigmentation.

Materials and Methods

Organisms

Laurentiella acuminata (Fedriani et al. 1976), a hypotrichous ciliate, was isolated from a sample of water collected at "Parque de Maria Luisa" (Sevilla). The green alga *Chlorogonium* sp. was kindly supplied by Prof. Ammermann (Tübingen University), the blue-green alga *Anacystis nidulans* (strain L 1402-1) was obtained from Göttingen University's Algal Culture Collection

Culture Conditions

The cultures of the ciliated protozoon *Laurentiella acuminata* were maintained at $20 \pm 1°C$ in Pringsheim's medium and fed on the unicellular green alga *Chlorogonium* sp. The algae used were grown autotrophically under white light ($25\ W \times m^{-2}$) at 30°C on 5% CO_2 in air in the culture medium of Arnon et al. 1974 for *A. nidulans* and in the Pringsheim's medium for *Chlorogonium* sp.

In order to obtain the resting cysts, vegetative cells were transferred from the maintenance ulture to fresh Pringsheim's medium in the absence of algae.

Pigment Extraction

Resting cysts were harvested by centrifugation (3000 g for 5 min), washed and resuspended cn 0.05 M phosphate buffer, pH 7.0, and then ruptured by sonication (20 Kc/s, 50–60 W) for 15 min. Unbroken cells and cyst walls were removed by centrifugation (20 000 g for 20 min). The supernatant was then lyophilized. Extraction of the pigments was carried out using hexane as organic solvent. About three ml of this solution was applied to an alumina column (3 × 10 cm) equilibrated with hexane. The column was washed with a serial solvents of increasing polarity (hexane, benzene, ethanol and methanol). Fractions with absorption at 440 nm were pooled and further used for the measurements.

Spectrophotometric Methods

The *in vivo* spectra were obtained by microspectrophotometry using a microdensitometer interferometer Vickers-M85. For the visible spectrophotometry a Beckman-Acta III spectrophotometer was used. The fluorescence was measured in an Aminco-Bowman spectrofluorimeter.

Chemicals

An alcoholic solution (1% w/v) of vitamin A was used emulsified in polyoxyethylenesorbitan (Tween-80), at concentrations ranging from 10 to 500 ppm. Nicotine, an inibitor of the carotenogenesis, was used at 6 mM concentration. Vitamin A, nicotine and Tween-80 were obtained from Sigma Chemical Co.

Results

The *in vivo* absorption spectra along the six steps from vegetative cell to resting cyst presented the following characteristics: In stage V the spectrum was similar to that of the alga (*Chlorogonium* sp.) normally used as the ciliate food. The next stage (stage A) was characterized by the gradual disappearance of food vacuoles. This fact was simultaneous with the decrease in the maximum of absorption at 650 nm. In stage B (Fig. 1), the complete disappearance of the pigmentation was noticed by absence of absorption on the visible range. The following stages (C and D) showed a progressive increase in absorption of the zone from 400 to 500 nm. The *in vivo* absorption spectrum during the last stage (stage E) remained similar to that of a carotenoid pigment (Fig. 1).

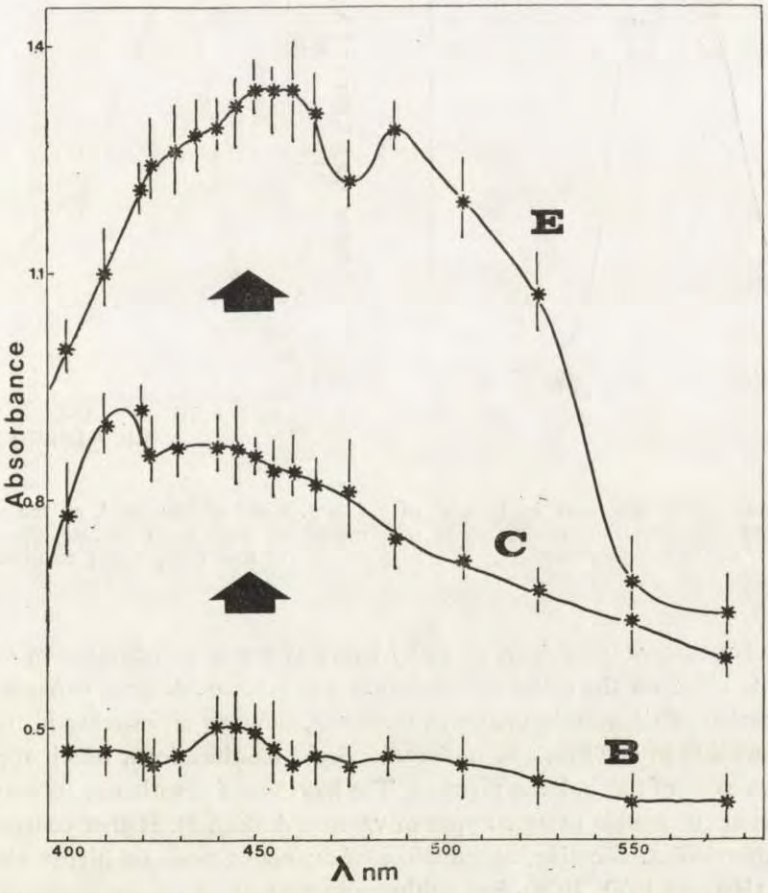

Fig. 1. *In vivo* absorption spectra of cells in the stages B, C and E. Each point indicates average value of four independent measures

Using hexane as a solvent, a fat-soluble pigment was extracted from the resting cysts which showed a visible absorption spectrum with maxima at 420, 440 and 466 nm (Fig. 2).

An estimate of the quantity of the cystic pigment, referred to cellular dry weight, showed a value of about 110 ppm.

The green alga *Chlorogonium* sp. and the blue-green alga *Anacystis nidulans*, both foods for the protozoon, show similar pigments. The spectra of cysts obtained from vegetative cells fed with both types of algae present very similar maxima of absorption.

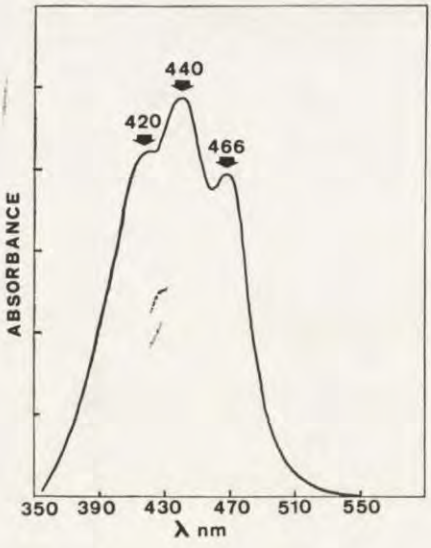

Fig. 2. Absorption spectrum in hexane of the isolated pigment from resting cysts of *Laurentiella acuminata*

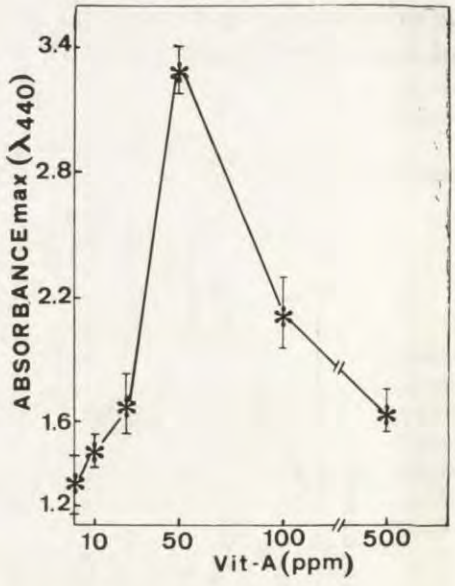

Fig. 3. Effect of vitamin A on the cystic pigmentation. Each point indicates average value of four independent measures

Knowing that vitamin A is, in many living systems, an activator of carotenogenesis, its effect on the cystic pigmentation was studied. A great enhancement of the coloration of the resting cysts was observed, showing an increase in the absorbance from 400 to 600 nm, and undergoing light modifications, which approaches to the spectrum of the isolated pigment. The increase of absorbance at wavelength 440 nm is about double using 50 ppm of vitamin A (Fig. 3). Higher concentrations have a lower effect. Nicotine, an inhibitor of carotenogenesis on higher plants and bacteria (Howes 1970, 1974), has neither apparent effect on the cystic pigmentation, nor on the intensity or the shape of the spectrum.

Discussion

It is known that there is a degradative process during the encystment. Thus, the decrease in the maximum absorption at 650 nm found in the present experiment could be possibly due to a chlorophyll degradation. The pigment isolated from the resting cysts presents characteristics of α-carotene (Moss and Weedon 1976) or a derivative from it. The great similarity between the spectrum of the isolated pigment and the spectra observed *in situ* during the last stages of the encystment showed that it may probably be the principal reason for the cystic coloration.

In many living systems, vitamin A can be an activator of carotenogenesis or a substrate of that. The observation that vitamin A, at very low concentrations, does not produce effects on the cystic pigmentation can preclude its action as a catalyst. The maximum of absorbance at 440 nm has been obtained using 50 ppm of vitamin A, suggesting its possible use as a substrate. On the other hand, nicotine has not inhibitory effect on the pigmentation, indicating that the process can be an alteration of precursors rather than a *de novo* synthesis. The above observations and the similarity between the algal carotene and that of the resting cyst suggest an active accumulation from material of algal origin, which may be the cause of the pigmentation.

Plants as well as many fungi and bacteria synthesize carotenoids *de novo*, but animals do not. In animals, carotenoids are obtained from the diet and then enzymatically altered (Harding and Shropshire 1980, Fox 1979). Then it can be suggested that the cystic pigment may be obtained from the algae, which are the habitual food of the protozoon, and then an accumulation of it can occur in the encysted cell. This accumulation could not be observed during the stages A and B.

The resting cystic pigmentation of this ciliate may have a protective function before the photooxidation originated by an excess of light on the desiccated natural medium. The desiccation is often an encystment inductor. Furthermore, a possible mimetic function may exist. In this sense, a protective function for carotenoids has been proposed for non-photosynthetic organisms (Feldman and Linstrom 1964).

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Сравнительное исследование антигенного состава инфузорий *Colpoda maupasi*

The Comparative Investigation of Antigenic Content of Ciliates *Colpoda maupasi*

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Синопсис. Изучено изменение антигенного спектра клеток инфузорий *Colpoda maupasi* — трофонтов и цист покоя — в нормальных условиях культивирования и цист, испытавших действие замораживания при —196°С и коротковолнового УФ облучения. Результаты, полученные с нативными иммуносыворотками с помощью реакций иммобилизации и агглютинации, позволяют сделать вывод о сходстве состава поверхностных антигенов трофонтов и цист покоя инфузорий. Замораживание вызывает значительные изменения поверхностных антигенов цист, а УФ облучение не изменяет спектр поверхностных антигенов. С помощью иммунодиффузных методов удалось показать, что кроме общих растворимых антигенов на стадии трофонтов и цист покоя существует антиген, характерный только для стадии цист. Удалось также установить изменение антигенного набора цист после действия экстремальных факторов. Показано антигенное упрощение и появление нового антигена после замораживания, а также появление по крайней мере двух антигенов, не отмеченных в интактных цистах, после облучения.

Применение иммунологических методов исследования, характеризующихся высокой чувствительностью и уникальной специфичностью, оказалось удачным для описания свойств клеток простейших и их сопоставления. К настоящему времени уже накопился важный фактический материал, полученный при использовании иммунологических методов. Важным следствием всех этих исследований явился вывод о том, что серотипы инфузорий могут быть изменены физическими факторами, такими как температура, УФ излучение,

изменение питания, обработка ферментами и антибиотиками (Wagtendonk 1951, Beale 1953, Sonneborn et al. 1953, Austin et al. 1956, Margolin et al. 1959, Sikora 1966). Некоторые авторы высказали предположение о том, что определенному физиологическому или физическому состоянию клетки соответствует строго определенный набор антигенов (Kimball 1964, Sommerville 1969). Если это допущение справедливо, то оно позволяет изучать динамику антигенов простейших при различных изменениях клеточного метаболизма, например при действии повреждающих факторов. Между тем подобные попытки пока единичны.

В нашей работе сделана попытка охарактеризовать с помощью методов иммунологии изменение антигенного спектра клеток инфузорий, взятых на разных стадиях жизненного цикла в нормальных условиях культивирования и клеток, испытавших действие экстремальных факторов: глубокого охлаждения и УФ облучения.

Материал и методика

Работа проведена на культуре инфузорий *Colpoda maupasi*, изученных ранее в отношении действия экстремальных факторов (Lozina-Lozinsky and Uspenskaya 1968, Успенская 1971). Инфузории культивировались на минеральной среде Лозина-Лозинского в аксеничных условиях с использованием смеси антибиотиков при температуре 23°C.

Материалом для иммунологического исследования послужили две стадии жизненного цикла — трофонты и цисты покоя, а также цисты покоя, подвергнутые УФ облучению и глубокому замораживанию. Трофонты использовались в конце логарифмической фазы роста из массовой культуры. Для получения цист покоя массовая культура клеток выдерживалась без смены среды в течение 7 суток. К этому времени процесс инцистирования заканчивался, и отсюда отсчитывался возраст цист. В опытах использовали 14-суточные цисты. Клетки инфузорий получали в большом количестве, промывали стерильной средой несколько раз и отделяли от нее центрифугированием при 4000 g в течение 15 мин. Конечную концентрацию клеток определяли в камере Горяева.

В качестве антигенов использовали живые клетки, соответствующие данным стадиям жизненного цикла инфузорий, клетки, подвергнутые действию замораживания и УФ-облучения, а также водно-солевые экстракты тех же клеток.

Для замораживания применялся жидкий азот (−196°C). Цисты замораживались на бактериологических фильтрах в тонкостенных бюксах, которые устанавливались на медную площадку, плотно соединенную с цилиндром, помещенным в жидкий азот. Скорость охлаждения всегда была постоянной и составляла 1°/мин. Цисты находились в замороженном состоянии 3 ч, а затем оттаивались в водяной бане при температуре 60° до исчезновения видимых кристаллов льда. Такой режим замораживания–оттаивания позволяет получить 80–90% жизнеспособных цист после холодового воздействия. Затем цисты смывались с фильтров.

Для УФ-облучения служила лампа БУВ-15, 85% энергии излучения которой приходится на линию 254 nm. Цисты облучались постоянной дозой 7000 erg/mm² при оптимальной температуре культивирования. После облучения этой дозой выживаемость составляла 80–85%.

При использовании живых клеток в качестве тест-объекта в опыт брали от 150 до 200 живых организмов. С целью получения водно-солевых экстрактов соответствующие клетки смешивали 1 : 1 с 0.14 M раствором NaCl и гомогенизировали. Полученный гомогенат центрифугировали при 3000 g в течение 20 мин и надосадочную жидкость, содержащую растворимые антигены, консервировали мертиолатом. Содержание белка в антигенных препаратах определяли непосредственно перед опытом по биуретовой реакции, после чего концентрация белка во всех препаратах выравнивалась посредством соответствующего разведения 0.14 M раствором NaCl. Как правило, в опытах рабочая концентрация экстракта составляла 12 mg/ml белка, но в некоторых случаях была использована вдвое большая концентрация экстрактов.

В работе использовали 4 иммуносыворотки: против трофонтов, против цист покоя, против цист, подвергнутых глубокому охлаждению, и против цист, испытавших УФ-воздействие.

Для получения иммуносывороток кроликов иммунизировали по схеме (Sonneborn 1950), состоящей из 6 внутривенных инъекций соответствующего антигенного препарата — в краевую вену уха с интервалами в 3 суток в течение 3 недель. Концентрация препарата в каждой последующей инъекции увеличивалась на 10^6 клеток, т. е. вводили от 10^6 в первой инъекции до 7×10^6 — в последней, что составляло 3 mg белка за весь цикл иммунизации. Через месяц кроликов реиммунизировали посредством двух инъекций по 4×10^6 клеток. Кровь у кроликов брали на 8-й и 14-й день после последней инъекции. Нормальные кроличьи сыворотки, служившие контролем, получали из тех же кроликов перед иммунизацией.

В опытах были использованы нативные и концентрированные иммуносыворотки. Последние представляют собой гамма-глобулиновые фракции иммуносывороток, выделенные спиртовым методом по Кону (Зильбер и Абелев 1962), при этом все полученные таким способом антисыворотки концентрировали в 6 раз. Изучение антигенных свойств живых клеток — трофонтов и цист покоя — проводили с использованием нативных иммуносывороток и тест-объектов, учитывая реакции на цитотоксичность: иммобилизацию, агглютинацию и лизис. Для этого равные объемы последовательно разведенной иммуносыворотки и тест-объекта помещали на пластинки с углублениями. Действие иммуносыворотки учитывали в интервалах: 15 мин, 30 мин, 2 ч и 24 ч. Наблюдения вели с фазовым контрастом в темном поле. Каждая серия опытов проводилась в 3–5 повторностях.

Антигенные свойства клеток инфузорий были изучены также с помощью реакции преципитации в агаре и реакции специфической задержки преципитации в агаре по Бьерклунду в их модификации на предметном стекле.

Результаты и обсуждение

Изучение антигенного состава клеток инфузорий *Colpoda maupasi* на разных стадиях жизненного цикла, а также при воздействии экстремальных факторов на цисты покоя было проведено с помощью нативных и концентрированных иммуносывороток. Нативные иммуносыворотки использовались для постановки реакций иммобилизации и агглютинации с целью выявления поверхностных антигенов, а концентрированные иммуносыворотки использовались в реакции преципитации в агаре (РПА) и реакции специфической задержки преципитации для определения водорастворимых, преимущественно цитоплазматических антигенов.

Рассмотрим вначале результаты, полученные с нативными иммуносыворотками, при сравнительном изучении антигенного спектра инфузорий соответствующих стадий жизненного цикла в нормальных условиях культивирования.

Иммуносыворотки против трофонтов и цист покоя были исследованы в прямых и перекрестных реакциях с соответствующими тест-объектами (Табл. 1 и 2). Иммуносыворотка против трофонтов отличалась высокой активностью, обнаруживая реакции иммобилизации и агглютинации с антигенами трофонтов в разведении 1 : 640 (Табл. 1). При смешивании трофонтов с гомологичной иммуносывороткой наблюдались весьма характер-

Таблица 1
Table 1

Активность иммуносыворотки против трофонтов инфузорий при взаимодействии с трофонтами

Activity of immunoserum to trophontes of ciliates with trophontes

Разведение иммуносыворотки Immunoserum dilution	Процент клеток, обнаруживших реакции: Percentage of cells showing:									
	иммобилизации immobilization					агглютинации agglutination				
	время наблюдения time of observation					время наблюдения time of observation				
	15 мин 15 min	30 мин 30 min	2 ч 2 h	4 ч 4 h	24 ч 24 h	15 мин 15 min	30 мин 30 min	2 ч 2 h	4 ч 4 h	24 ч 24 h
1 : 10	70	100	100	100	100	70	70	100	100	100
1 : 20	50	70	100	100	100	50	50	60	60	70
1 : 40	10	60	70	70	70	10	50	55	60	60
1 : 80	0	10	50	50	50	0	0	0	30	50
1 : 160	0	0	0	30	50	0	0	0	20	30
1 : 320	0	0	0	0	30	0	0	0	0	10
1 : 640	0	0	0	0	10	0	0	0	0	5

ные реакции. Сначала движение инфузорий ускоряется и становится беспорядочным. Затем оно замедляется, хотя реснички еще активны. Следующая стадия характеризуется прекращением движения ресничек, но еще некоторое время наблюдается циркуляция цитоплазмы, а затем наступает гибель клеток. Быстрота, с которой происходят эти процессы, зависит от концентрации иммуносыворотки: при слабых разведениях иммуносывороток реакции иммобилизации и агглютинации наблюдались в течение 2 ч, при больших — в течение суток. При использовании антисыворотки в больших разведениях реакции сильно растянуты во времени, поэтому факт иммобилизации и агглютинации в этих случаях установить довольно трудно. Большинство обез-

Таблица 2
Table 2

Активность иммуносыворотки против интактных цист инфузорий при взаимодействии с трофонтами

Activity of immunoserum to intact cysts of ciliates with trophontes

Разведение иммуносыворотки Immunoserum dilution	Процент клеток, обнаруживших реакции: Percentage of cells showing:					
	иммобилизации immobilization			агглютинации agglutination		
	время наблюдения time of observation			время наблюдения time of observation		
	2 ч 2 h	4 ч 4 h	24 ч 24 h	2 ч 2 h	4 ч 4 h	24 ч 24 h
1 : 10	0	100	100	0	80	100
1 : 20	0	0	70	0	0	60
1 : 40	0	0	50	0	0	45
1 : 80	0	0	35	0	0	10
1 : 160	0	0	10	0	0	0

движенных организмов погибает через короткое время, и лишь небольшая часть инфузорий (от 5 до 10%) восстанавливает свою подвижность или образуются цисты покоя, из которых впоследствии можно было получить вполне жизнеспособные организмы. Подобное действие иммуносыворотки на „свои" клетки было обнаружено у парамеций (Beale 1957). Следует заметить, что нормальная кроличья сыворотка в последовательных разведениях не обнаруживала указанных реакций с исследованными тест-объектами. Иммуносыворотка против трофонтов не реагировала с цистами покоя.

Иммуносыворотка против цист покоя показала меньшую активность по сравнению с иммуносывороткой против трофонтов, обнаруживая реакции иммобилизации и агглютинации с трофонтами в разведении 1 : 160. При этом полная агглютинирующая и иммобилизирующая активность антисыворотки проявлялась в течение суток (Табл. 2). Лишь при разведении сыворотки 1 : 10 иммобилизирующий эффект был максимальным и проявлялся в течение 4 ч. Спустя сутки при всех разведениях иммуносыворотки, и даже в минимальном разведении, обездвиженные инфузории восстанавливали нормальное движение или образовывали жизнеспособные цисты покоя.

При смешивании иммуносыворотки против цист с гомологичным тест-объектом мы не обнаружили агглютинирующего действия (Табл. 3). Однако при использовании неразведенной нативной иммуносыворотки можно было наблюдать литическое действие антисыворотки на цисты покоя. Напомним,

Таблица 3
Table 3

Активность иммуносывороток при взаимодействии с тест-объектами

Activity of immunosera with test objects

Иммуносыворотки Immunoserum	Тест-объекты Test objects								
	Трофонты Trophontes			Интактные цисты Intact cysts		Замороженные цисты Frozen cysts		Облученные УФ цисты UV-Irradiated cysts	
	иммобилизация immobilization	агглютинация agglutination	лизис lysis	агглютинация agglutination	лизис lysis	агглютинация agglutination	лизис lysis	агглютинация agglutination	лизис lysis
Против трофонтов Anti-trophontes serum	1 : 640	1 : 320	— —	— —	— —	— —	— —	1 : 10	— —
Против интактных цист Anti-intact cysts serum	1 : 160	1 : 80	— —	— —	неразведенная non-diluted	— —	— —	— —	— —
Против замороженных цист Anti-frozen cysts serum	1 : 40	— —	— —	— —	— —	— —	1 : 40	— —	— —
Против облученных УФ цист Anti-UV-irradiated cysts cerum	1 : 160	1 : 80	— —	— —	— —	— —	— —	1 : 10	неразведенная non-diluted

Примечание. Численные значения показывают наибольшее разбавление иммуносыворотки, при котором еще наблюдается реакция. (— —) означает отсутствие реакции

Notes. Numbers indicate the largest dilution of immunoserum when the reaction is proceeding. (— —) indicates no reaction.

что неразведенная иммуносыворотка против трофонтов не обнаруживала литического действия со всеми исследуемыми тест-объектами.

Представленные данные позволяют сделать вывод о сходстве состава поверхностных антигенов трофонтов и цист покоя инфузорий на основании аналогичного характера реакций иммобилизации и агглютинации. Кроме того, на основании полученных результатов можно, по-видимому, сделать заключение о том, что в антигенном спектре исследуемых клеток имеются количественные различия. С помощью меченых антител удалось показать, что ,,иммобилизационный антиген" локализуется на пелликуле и ресничках инфузорий (Beale 1957, Beale and Kačser 1957, Beale and Mott 1962). Очевидно, что антигены, ответственные за иммобилизирующее и агглютинирующее действие, присущи как стадии трофонтов, так и стадии цист покоя что было показано ранее (Margolin et al. 1959, Padnos 1962).

На следующем этапе работы было проведено сравнение антигенного спектра интактных цист с таковым цист, подвергнутых действию замораживания и УФ облучения.

Полученная нативная иммуносыворотка против замороженных цист обладала очень низкой активностью. Прежде всего это выявилось при действии на трофонты. Реакция иммобилизации сильно растянута во времени — она проявляется лишь в течение суток. Наибольшее разведение, при котором реакция еще проявляется — 1 : 40, дальнейшее разведение антисыворотки приводило к потере ее активности. Реакция агглютинации в этом случае не была выявлена даже при использовании неразбавленной сыворотки. Отсутствие реакции агглютинации было отмечено также и при действии указанной иммуносыворотки на интактные цисты. Агглютинирующий эффект антисыворотки против замороженных цист не проявлялся и при взаимодействии со ,,своими" антигенами, т. е. с замороженными цистами. Однако при смешивании данной иммуносыворотки с замороженными цистами наблюдалось довольно сильное литическое действие: сыворотка в разведении 1 : 40 еще обнаруживала эту реакцию, причем завершалась она быстро — в течение 2–4 ч.

Если сравнить между собой действие двух сывороток: против интактных цист и против замороженных цист, то оказывается, что имеют место значительные различия (Табл. 3). Это может быть легко объяснимо, если иметь в виду, что повреждающее действие замораживания при температуре —196° главным образом связано с поверхностными структурами клетки. Нужно отметить, что даже в тех случаях, когда повреждение под действием глубокого охлаждения наименьшее (именно такого эффекта мы добивались в нашем случае при использованном режиме замораживания–оттаивания), могут происходить нарушения в мембране клеток и молекулярные перестройки белков (Лозина-Лозинский 1972). Эти повреждения поверхностных структур, вероятно, отразились на характеристике спектра поверхностных антигенов,

выявляемых с помощью реакций иммобилизации и агглютинации с нативными сыворотками.

Иммуносыворотка против цист, подвергнутых УФ облучению, при взаимодействии с трофонтами обнаруживала реакции иммобилизации и агглютинации таким образом, что титр данной иммуносыворотки оказался сходным с титром иммуносыворотки против интактных цист. Реакции иммобилизации и агглютинации проявились при действии на трофонты как одной, так и другой сыворотки в одинаковых разведениях (Табл. 3). Эти результаты заметно отличаются от результатов, полученных с иммуносывороткой против цист, подвергнутых замораживанию, указывая на своеобразие действия замораживания и УФ облучения на поверхностные антигены цист. Известно, что в отличие от действия замораживания, когда прежде всего повреждаются поверхностные структуры, первичной мишенью для УФ-лучей являются внутриклеточные структуры простейших и именно ДНК (Calkins 1964).

Иммуносыворотка против облученных УФ цист не давала реакции агглютинации с интактными цистами. Однако эта же сыворотка при действии на гомологичный тест-объект, т. е. на облученные цисты выявляла реакцию агглютинации в разведении 1 : 10. При этом цисты открепляются от субстрата и склеиваются в небольшие группы. Помимо этого, при взаимодействии неразведенной иммуносыворотки с облученными цистами проявлялось ее лизирующее действие.

Таким образом, результаты исследования поверхностных антигенов трофонтов, интактных цист и цист после воздействия экстремальных факторов показали, что на поверхности трофонтов имеются антигены, участвующие в реакциях иммобилизации и агглютинации. В интактных цистах эти антигены, по-видимому, также присутствуют, но на данной стадии жизненного цикла инфузорий, вероятно, срабатывают механизмы, частично блокирующие поверхностные антигены, и как следствие этого — отсутствие реакции агглютинации при взаимодействии иммуносыворотки против интактных цист с гомологичным тест-объектом. Однако при введении интактных цист в организм животного в антисыворотке можно обнаружить антитела против указанных поверхностных антигенов. Свидетельством этому является реакция иммуносыворотки против интактных цист с трофонтами.

Замораживание вызывает, очевидно, значительные изменения поверхностных антигенов цист: иммуносыворотка против замороженных цист в значительно меньшем титре дает реакцию иммобилизации с трофонтами и совсем не проявляет агглютинирующего эффекта. Иная картина наблюдается при УФ облучении цист. Иммуносыворотка против облученных цист дает реакцию агглютинации со „своими" цистами. Этот факт можно объяснить действием УФ облучения на механизмы, частично освобождающие поверхностные антигены, которые способны вступать в реакцию агглютинации и синтез которых в интактных цистах заблокирован. Подтверждением этого может служить

агглютинирующее действие иммуносыворотки против трофонтов на облученные цисты (см. Табл. 3).

Далее рассмотрим результаты исследования водо-растворимых антигенов, полученных с помощью иммунодиффузных методов при использовании концентрированных иммуносывороток.

Остановимся вначале на результатах сравнительного изучения антигенного состава клеток инфузорий *C. maupasi* на разных стадиях жизненного цикла в нормальных условиях культивирования. В прямой постановке РПА иммуносыворотки против трофонтов и цист покоя тестировали с экстрактом клеток (гомологичным и гетерологичным) этих стадий жизненного цикла инфузорий (Табл. 4). Иммуносыворотка против трофонтов реагировала с экс-

Таблица 4
Table 4

Результаты реакции преципитации иммуносывороток против трофонтов и интактных цист инфузорий с исследуемыми тест-экстрактами

Results of direct precipitation reaction of immunosera to trophontes and intact cysts with test extracts

Иммуносыворотка Immunoserum	Тест-экстракты Test-extracts	
	трофонты trophontes	интактные цисты intact cysts
Против трофонтов Anti-trophontes serum	++++	++
Против интактных цист Anti-intact cysts serum	++	+++

Примечание. В этой и следующих таблицах: плюс – одна полоса преципитации, минус – отсутствие реакции преципитации.

Notes: In this and following tables: plus – one band of precipitation, minus – the absence of precipitation reaction.

Таблица 5
Table 5

Результаты реакции специфической задержки преципитации тест-экстрактов трофонтов и интактных цист инфузорий с иммуносыворотками

Results of the specific inhibition of precipitation reaction of immunosera to trophontes and intact cysts with test extracts

Иммуносыворотка Immunoserum	Тест-экстракты Test-extracts	
	трофонты trophontes	интактные цисты intact cysts
Против трофонтов, истощенная экстрактом цист Anti-trophontes serum, absorbed with extract of cysts	–	–
Против цист, истощенная экстрактом трофонтов Anti-cysts serum, absorbed with extract of trophontes	–	+

Обозначения см. Таблица 4.
Explanations see Table 4.

трактом трофонтов, образуя 4 полосы преципитации, и с экстрактом цист, образуя 2 полосы. В то же время иммуносыворотка против цист давала со „своим" экстрактом (экстрактом цист) 3 полосы преципитации и 2 полосы с экстрактом трофонтов. При этом следует отметить, что по крайней мере два антигена являются общими для данных стадий инфузорий, о чем свидетельствует феномен идентичности полос преципитации, выявляемый реакциями иммуносывороток с исследуемыми экстрактами. Результаты прямой постановки РПА свидетельствуют кроме того о различиях антигенного состава трофонтов и цист покоя, о чем можно судить по количеству полос преципитации, выявляемых иммуносыворотками с гетерологичными тест-экстрактами. Однако остается неясным вопрос о характере этих различий: изменяется ли антигенный состав качественно или отличия лишь в количестве антигенов. На этот вопрос мы смогли ответить, используя реакцию специфической задержки преципитации в агаре, перекрестно истощая иммуносыворотки гетерологичными экстрактами.

Путем предварительного титрования была подобрана концентрация экстрактов (трофонтов и цист), при помощи которой удавалось полностью извлечь из гомологичной иммуносыворотки антитела против „своих" антигенов. Эти концентрации экстрактов в дальнейшем были использованы для перекрестного истощения иммуносывороток.

В лунку, предназначенную для иммуносыворотки против цист, предварительно помещали экстракт трофонтов и после полной его диффузии в агаре (обычно через 18–20 часов при комнатной температуре) в ту же лунку заливали иммуносыворотку. Затем тестировали с экстрактами трофонтов и цист покоя. При этом иммуносыворотка против цист не реагировала с экстрактом трофонтов и продолжала выявлять 1 полосу преципитации с экстрактом цист. Повышение в 1.5–2 раза концентрации экстракта трофонтов для истощения иммуносыворотки не привело к какому-либо снижению степени реакции иммуносыворотки со „своим" экстрактом, что свидетельствует очевидно не о количественном преобладании данного антигена в цистах. По-видимому не обнаруженный в трофонтах антиген можно считать характерным для стадии цист покоя инфузорий.

При истощении иммуносыворотки против трофонтов экстрактами цист удалось подобрать концентрацию истощающего экстракта, при которой иммуносыворотка не реагировала как с экстрактом цист, так и с экстрактом трофонтов. Это свидетельствует о том, что антигенный состав трофонтов и цист имеет общие антигены, а результаты, полученные в РПА с этой иммуносывороткой отражают количественные различия (Табл. 5).

Таким образом, с помощью иммунодиффузных методов удалось показать, что на стадии трофонтов и цист, кроме общих растворимых антигенов для данных стадий развития инфузорий, обнаружен антиген, характерный по-видимому только для цист.

Далее такой же анализ был проведен при изучении цист, подвергнутых воздействию замораживания и УФ облучения. С помощью РПА иммуносыворотка против интактных цист выявляла в экстрактах цист три антигена, тогда как в экстрактах цист, подвергнутых глубокому охлаждению, эта же сыворотка выявляла лишь два антигена. Эти два антигена оказались идентичными двум из трех антигенов, выявленных указанной иммуносывороткой в экстрактах интактных цист, о чем можно судить по феномену идентичности полос преципитации. В то же время иммуносыворотка против замороженных цист образует 4 полосы преципитации с гомологичным тест-экстрактом и 3 полосы — с экстрактом интактных цист. Эти результаты свидетельствуют о том, что в антигенном спектре интактных цист и цист, подвергнутых замораживанию, имеются общие антигены, но кроме того, очевидны и различия (Табл. 6).

Подтверждением сказанному могут служить результаты, полученные при использовании реакции специфической задержки преципитации. Иммуносыворотку против интактных цист истощали экстрактом замороженных цист.

Таблица 6
Table 6

Результаты реакции преципитации иммуносывороток против интактных цист, против замороженных цист и против облученных УФ цист инфузорий с исследуемыми тест-экстрактами

Results of direct precipitation reactions of immunosera to intact cysts, frozen cysts and UV-irradiated cysts of ciliates with test extracts

Иммуносыворотка Immunoserum	Тест-экстракты Test extracts		
	замороженные цисты frozen cysts	облученные УФ цисты UV-irradiated cysts	интактные цисты intact cysts
Против замороженных цист Anti-frozen cysts serum	++++	−	+++
Против облученных УФ цист Anti-UV-irradiated cysts serum	−	+++++	+++
Против интактных цист Anti-intact cysts serum	++	+++	+++

Обозначения см. Таблица 4.

Explanations see Table 4.

В этом случае антисыворотка продолжала реагировать с экстрактом нормальных цист с образованием одной полосы преципитации, но не давала реакции с экстрактами замороженных цист (Табл. 7). Следовательно, можно заключить, что в цистах, подвергнутых замораживанию, отсутствует или в значительной мере снижен синтез одного из антигенов, характерных для антигенного набора интактных цист.

Следующая серия опытов была проведена с иммуносывороткой против замороженных цист. Эту иммуносыворотку истощали экстрактом интакт-

Таблица 7
Table 7

Результаты реакции специфической задержки преципитации тест-экстрактов интактных цист и замороженных цист с иммуносыворотками

Results of the specific inhibition of precipitation reactions of antisera to intact cysts and frozen cysts with test extracts

Иммуносыворотка Immunoserum	Тест-экстракты Test extracts: интактные цисты intact cysts	Тест-экстракты Test extracts: замороженные цисты frozen cysts
Против интактных цист, истощенная экстрактом замороженных цист Anti-intact cysts serum, absorbed with extracts of frozen cysts	+	−
Против замороженных цист, истощенная экстрактом интактных цист Anti-frozen cysts serum, absorbed with extracts of intact cysts	−	+

Обозначения см. Таблица 4.

Explanations see Table 4.

Таблица 8
Table 8

Результаты реакции специфической задержки преципитации тест-экстрактов интактных цист и облученных УФ цист с иммуносыворотками

Results of the specific inhibition of precipitation reactions of antisera to intact cysts and UV-irradiated cysts with test extracts

Иммуносыворотка Immunoserum	Тест-экстракты Test extracts: интактные цисты intact cysts	Тест-экстракты Test extracts: облученные УФ цисты UV-irradiated cysts
Против интактных цист, истощенная экстрактом облученных УФ цист Anti-intact cysts serum absorbed with extract of UV-irradiated cysts	−	−
Против облученных УФ цист, истощенная экстрактом интактных цист Anti-UV-irradiated cysts serum absorbed with extract of intact cysts	−	++

Обозначения см. Таблица 4.

Explanations see Table 4.

ных цист и тестировали также с гомологичными и гетерологичными экстрактами цист. При этом иммуносыворотка не реагировала с экстрактом интактных цист, но в то же время проявляла одну полосу преципитации с экстрактом замороженных цист (Табл. 7). Повышение концентрации истощающего экстракта не влияло на характер выраженности реакции преципитации. Полученные результаты позволяют говорить не о количественном преобладании обнаруженного в замороженных цистах антигена, а по-видимому, о проявлении в них такого антигена, который не удалось выявить в интактных цистах.

Далее рассмотрим результаты сравнительного изучения антигенного состава водно-солевых экстрактов интактных цист и цист, облученных УФ лучами (Табл. 6). В РПА с помощью иммуносыворотки против интактных цист мы сопоставили антигенные составы указанных цист. При этом не удалось обнаружить каких-либо различий в антигенном наборе исследуемых цист: иммуносыворотка проявляла 3 полосы преципитации как с экстрактами интактных цист, так и с экстрактами цист после УФ облучения. Иной результат был получен при использовании иммуносыворотки против облученных цист. Эта антисыворотка выявляла три антигена в экстрактах интактных цист и пять антигенов в экстрактах облученных цист, причем три из них оказались идентичными антигенами интактных цист.

Таким образом, в условиях наших экспериментов удалось установить различия антигенного набора интактных и облученных УФ цист инфузорий. Однако ответить на вопрос о характере этих различий, как и в опытах с замороженными цистами, удалось, используя реакцию специфической задержки преципитации в агаре перекрестно истощая имеющиеся в нашем распоряжении антисыворотки (табл. 8).

При истощении иммуносыворотки против интактных цист экстрактом облученных цист оказалось, что истощающая доза экстракта (12 mg/ml белка) достаточна для полной нейтрализации в иммуносыворотке антител против интактных цист, т. е. иммуносыворотка давала отрицательный результат со всеми исследуемыми тест-экстрактами. Это обстоятельство свидетельствует о том, что антигены, обнаруженные в интактных цистах, в той же мере представлены и в облученных цистах.

Следующая серия опытов была проведена с иммуносывороткой против облученных цист, которую истощали экстрактом интактных цист. В этом случае истощение иммуносыворотки оказалось неполным: иммуносыворотка не реагировала с тест-экстрактом интактных цист, но продолжала реагировать с тест-экстрактом облученных цист, образуя 2 полосы преципитации. Увеличение концентрации экстракта, взятого для истощения антисыворотки, не меняло характера реакции. Эти данные позволяют сделать заключение о том, что в цистах, подвергнутых УФ-воздействию, выявляются по крайней мере два антигена, не обнаруженые в интактных цистах.

Подводя итоги сравнительного изучения антигенного состава интактных цист и цист, подвергнутых воздействию экстремальных факторов, можно заключить, что были обнаружены изменения антигенного состава в цистах после их замораживания и УФ облучения. Антигенные особенности замороженных цист проявляются в том, что обнаружено антигенное упрощение, т. е. в антигенном наборе этих цист отсутствовал или в значительной мере был снижен синтез одного из антигенов, характерного для интактных цист. Кроме того, обнаружено появление в замороженных цистах антигена, наличие которого в интактных цистах не было установлено.

При изучении антигенного состава цист после УФ облучения не удалось выявить антигенного упрощения, однако в этих цистах появляются по крайней мере два антигена, отсутствующие в антигенном наборе интактных цист. Обсуждая эти данные, следует иметь в виду, что действие дозы 7000 erg/mm^2 не оказывает на цисты покоя сильного повреждающего действия, а напротив — приводит к стимуляции клеточного деления. Эта стимуляция выражается в повышении темпа размножения в течение 7 суток после облучения (Лозина-Лозинский и Успенская 1971). Данные Calkins (1968) свидетельствуют об инактивации некоторых клеточных ферментов даже под действием малых доз УФ облучения, связанной с разрушением при этом белковых комплексов, что в свою очередь является толчком для активации других ферментов, ответственных за процессы репарации в клетке. По-видимому, эти процессы приводят к усложнению антигенного спектра облученных цист.

SUMMARY

The antigenic composition of ciliates *Colpoda maupasi* was investigated using trophontes and resting cysts under normal cultivation, and cysts after cold (—196°) and short-waved UV exposition. The data obtained for the native immunosera with immobilization and agglutination reaction, suggest the similarity in the surface antigenic composition of trophontes and resting cysts. The deep freezing resulted in significant changes of surface antigen, whereas UV irradiation did not cause any notable shifts in the surface antigenic composition.

Using immunodiffusive methods, an antigen specific only for the cyst stage has been shown in trophontes and cysts in addition to general soluble antigens. Some antigenic changes were shown in cysts after the action of extreme factors. An antigenic simplification and a new antigen were revealed after freezing, at least 2 antigens from those not specific for the intact cyst being demonstrated after UV-irradiation.

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The Lytic Enzymes of Pathogenic and Non-pathogenic Strains of *Acanthamoeba castellanii* and *Naegleria fowleri*

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Synopsis. The comparison of the activity and localization of some lytic enzymes of amoebae was the object of this work. The following amoebae were investigated: *Acanthamoeba castellanii* pathogenic strain 309 and non-pathogenic strain Neff, *Naegleria fowleri* pathogenic strain Vitek and non-pathogenic strain 5 D. The results of the investigations showed that the pathogenicity of *Acanthamoeba castellanii* and *Naegleria fowleri* probably depends on the activity of acid hydrolases which can take part in the penetration of host cells.

Some free-living amoebae have particular properties: they are capable of penetrating into the organism of the host and multiplying in its tissues, mainly in its central nervous system. These invasions, as a rule, lead to meningoencephalitis ending with a fatal issue.

As can be seen from the paper by Kasprzak and Mazur (1972) the pathogenic strains of *Acanthamoeba* genus are widely spread and can be found in practically every water reservoir examined. The pathogenic strains of *Naegleria fowleri*, on the other hand, occur in an environment less commonly and their occurrence is connected with summer time and thermically contaminated water. At first, the *Naegleria fowleri* strains were only isolated from tissues or cerebrospinal fluid of infected individuals. Since 1972, the pathogenic strains of *Naegleria fowleri* have also been found in environment. In the last few years non-pathogenic variants of *Naegleria fowleri* have been obtained from similar, or the same water reservoirs (De Jonckheere et al. 1975, Kasprzak and Mazur 1978). In 1980, Stevens et al. described those variants as a new species of *Naegleria lovaniensis.*

Although the criteria of differentiating pathogenic strains from non-pathogenic ones in particular species of amoebae have become well known, not much has been

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found out so far about the mechanism of the pathogenic properties. The studies by Culbertson et al. (1968), Chang (1971) and Visvesvara and Callaway (1974) suggest that the amoebae may become capable of penetrating into the hosts tissues due to specialized cytolytic enzymes.

The opinion that hydrolytic enzymes condition the penetration of parasites into the hosts tissues has been supported by the results of studies on some of worms (Dresden and Asch 1972) and protozoa (Jarumulinta and Meagraith 1961, Meyer et al. 1958, Köberle 1972, Dvorak and Hyde 1973, von Brand 1973, Bongertz and Hungerer 1978).

The biochemical studies performed so far on lytic enzymes in amoebae and aimed at elucidating the pathogenesis of invasion have been conducted by the qualitative methods rather (Sargeaunt and Williams 1978, Nerad and Daggett 1979) than the quantitative ones. It seemed useful to find out the quantitative relations of the hydrolytic enzymes at different developmental stages of primarily free-living amoebae and the intracellular localization of those enzymes in pathogenic and non-pathogenic strains.

Material and Methods

Strains of amoebae: *Acanthamoeba castellanii*, strain 309, pathogenic for mice, isolated by Kasprzak and Mazur 1974, *Acanthamoeba castellanii*, strain Neff, non-pathogenic, isolated by Neff 1957, *Naegleria fowleri*, strain Vitek, pathogenic for mice, isolated by Červa et al. 1969, *Naegleria fowleri*, strain 5 D, non-pathogenic, isolated by Kasprzak and Mazur 1978. Having regard to the recently described non-pathogenic species of *Naegleria lovaniensis* (Stevens et al. 1980), it is to be stated that strain 5 D does not demonstrate the structures characteristic of this species.

Amoebal culture: The amoebae were cultured axenically in a liquid medium described by Červa (1966, 1969), composed of 2% Bacto-Casitone (Difco) and 10% serum. The amoebae of *Acanthamoeba* species were cultured at 24°C on medium containing an addition of horse serum, and of *Naegleria* species at 37°C on medium containing an addition of sheep serum.

Extraction of proteins: The amoebae harvested from 4–7 day cultures were centrifuged at 900 g for 5 min and subsequently washed three times with physiological salt solution. The sediment of protozoa thus obtained was suspended in extraction buffer 50 mM Tris-HCl, pH 7.4 at ratio 1 : 4. The cells were homogenized in teflon homogenizer at 4°C. The homogenates were centrifuged at 15,000 g for 30 min. For futher investigations were collected the supernatants which were stored in ice up to moment the determinations were carried out. The total protein content in the supernatants was determined by the method of Lowry et al. (1951).

Disc electrophoresis of proteins: The disc electrophoresis of proteins was performed in a Quickfit apparatus according to the method described by Davis and Ornstein (1959) and modified by Hadaś et al. (1977). The samples containing 200–400 μg of protein were placed on the top of the gel together with bromophenol blue marker. The electrophoresis was performed in 4°C for 60 min at 2.5 mA for each gel. The protein fractions thus separated were stained with Amido Black 10 B. The percentage taxonomic similarity of strains was calculated according to Whitney et al. (1968).

Enzymatic investigations: Proteolytic activity of protein in supernatants was determined after their electrophoretic distribution using the method of Herd and Motycka (1973). The ki-

netic determination of trypsin activity was performed according to the method of Erlanger et al. (1961). Acid and alkaline phosphatases were determined employing a method with p-nitrophenylophosphate ("Sigma 104") as substrate (Bessey et al. 1946). The isoenzymic composition of acid phosphatase was determined staining selectively enzymatic proteins after their electrophoretic distribution on polyacrylamide gel according to the Grogg and Pearse (1952) method. The isoenzymic composition of non-specific esterases was determined electrophoretically applying selective staining (Pearse 1961). The localization of acid phosphatase activity and that of non-specific esterase in amoebae in light microscope was performed by the method of Burck (1973).

Results

Electrophoretic distribution of protein fraction is presented in Pl. I 1. In *Acanthamoeba castellanii*, 20 protein fractions were revealed in pathogenic strain 309, and 19 in non-pathogenic strain Neff. No differences were observed in *Naegleria fowleri* between pathogenic strain Vitek and non-pathogenic 5 D, both the strains having 19 protein fractions.

The resemblance of proteins as calculated according to Whitney et al. (1968), between the strains of *Acanthamoeba castellanii* amounted to 95%; strain Neff did not have one fraction occurring in strain 309, while the remaining protein fractions were identical as regards their electrophoretic mobility. The staining intensity was different in particular fractions.

The proportional resemblance of protein between the strains of *Naegleria fowleri* amounted to 100%. All the fractions were identical as regards their electrophoretic mobility, but the staining intensity varied widely in particular fractions. The proportional resemblance of protein between *Acanthamoeba* and *Naegleria* amounted to 13%.

The proteolytic activity of proteins of free-living amoebae was found on the basis of clearings arising in the agar containing hyaluronic acid and horse serum due to digestion by proteinases. The pathogenic *Acanthamoeba castellanii* (309) and *Naegleria fowleri* (Vitek) strains were characterized by 5 fractions demonstrating the proteolytic activity, while the non-pathogenic *Acanthamoeba castellanii* (Neff) and *Naegleria fowleri* (5 D) ones by 4 fractions. The electrophoretic mobility of the proteins revealed was identical.

After 1 h incubation, it was possible to reveal the presence of proteolytic enzymes in one cathode and one anode fraction of the species studied, and after 24 h incubation the remaining fractions were revealed. The electrophoretic distribution of the proteases on agar gel is presented in Fig. 1.

The specific trypsin activity of amoebae in different periods of culture growth is shown in Table 1. The kinetic determinations of trypsin activity were performed spectrophotometrically in the supernatants obtained from the amoebae on the 4th, 5th and 7th day of their growth. Considerable differences in the trypsin activity

were observed according to the phase of growth, namely, in the logarithmic phase of growth (4–5 day culture) the trypsin activity in pathogenic strain was 2 to 10 times higher.

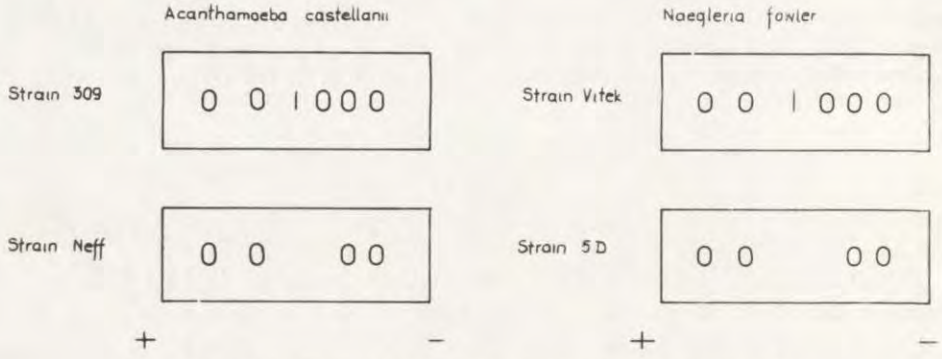

Fig. 1. The distribution of non-specific proteases on the agar gel

Table 1

The specific trypsin activity of amoebae in different periods of culture growth

Day of growth	*Acanthamoeba castellanii*				*Naegleria fowleri*			
	strain 309		strain Neff		strain Vitek		strain 5 D	
	mean value	S.D.	mean value	S.D.	mean value	S.D.	mean value	S.D.
4	7.4	1.1	0.2	0.2	16.2	2.4	14.0	1.4
5	16.4	2.3	2.3	0.5	35.0	5.0	16.5	2.4
7	16.0	1.8	14.8	1.5	16.6	2.8	18.2	3.6

The activities of acid and alkaline phosphatases were determined colorimetrically in the supernatants from homogenates of amoebae on the 4th and 7th day of their growth. The optimum of acid phosphatase activity is at pH 4.8 of 0.1 M citrate buffer and that of alkaline phosphatase activity at pH 9.8 of 0.1 M triethanolamine buffer. How the activities of acid and alkaline phosphatases are related with the day of culture growth is shown in Table 2. As can be seen, the activity of both the phosphatases in the logarithmic phase of growth is higher in pathogenic strains; the non-pathogenic strains were characterized by a much lower activity of those enzymes in this phase of culture growth.

The determination of isozymic composition of acid phosphatase was carried out on polyacrylamide gel. The electrophoretic distributions are presented in Pl. I 2. They show the presence of 5 isozymic fractions of acid phosphatase in both

Table 2

The activities of acid and alkaline phosphatases from homogenates of amoebae on the 4th and 7th day of their growth

Specification	Day of growth	*Acanthamoeba castellanii* strain 309		*Acanthamoeba castellanii* strain Neff		*Naegleria fowleri* strain Vitek		*Naegleria fowleri* strain 5 D	
		mean value	S.D.	mean value	S.D.	mean value	S.D.	mean value	S.D.
Acid phosphatase	4	17.8	3.3	4.5	1.0	18.8	3.2	4.2	1.1
	7	6.5	1.2	7.0	1.5	5.9	1.4	6.0	1.3
Alkaline phosphatase	4	7.5	1.3	4.0	1.0	3.8	0.9	1.6	0.6
	7	3.6	1.0	14.2	2.0	0.8	0.4	12.1	2.2

the pathogenic strain (309) and the non-pathogenic strain (Neff) of *Acanthamoeba castellanii*, and 8 fractions in the pathogenic strain (Vitek) and 5 fractions in the non-pathogenic strain (5 D) of *Naegleria fowleri*. The electrophoretic mobility of isoenzymic fractions of *Acanthamoeba castellanii* strains 309 and Neff was similar, but the strains differed considerably in their activities. The isozymic fractions of both the *Naegleria fowleri* strains were different as regards both the electrophoretic mobility and activity.

The isozymic composition of non-specific esterase is shown in Pl. I 3. The electrophoretic investigations revealed great heterogeneity of isozymes as regards the electrophoretic mobility and activity of fractions of *Acanthamoeba castellanii* strains 309 and Neff and *Naegleria fowleri* strains Vitek and 5 D. In the former 9 and in the latter 6 isozymic fractions of esterase were found.

The histochemical investigations on intracellular localization of acid phosphatase (Pl. II 4–7) revealed its uniform distribution in the cells of amoebae in the form of lead precipitates of various granularity. The pathogenic strains demonstrated greater activity of the enzyme in cells, so the enzymatic incubation of pathogenic amoebae was conducted for about 15 min. The enzymatic incubation of non-pathogenic amoebae was conducted for 20 min.

The histochemical investigations on intracellular localization of non-specific esterase revealed uniform distribution also of this enzyme in the cells of pathogenic and non-pathogenic strains of both the species studied (Pl. III 8–11).

Discussion

The electrophoretic analysis of proteins of amoebae revealed, as did previous studies on other protozoa, that the pathogenic and non-pathogenic strains within the same species demonstrate similar or identical protein patterns (Ebert 1973,

1974 a and b, Hadaś et al. 1977, Said-Fernandez et al. 1978, Sargeaunt and Williams 1978, Nerad and Daggett 1979). The differences, if any, mostly concerned the proportional contents of protein in particular fractions.

The analysis of protein composition of both *Acanthamoeba castellanii* strains revealed the absence of one fraction in Neff strain. Maybe the fractions of similar electrophoretic mobility, while running along each other and associating due to mutual interaction, gave in electrophorograms one common fraction of resultant electrophoretic mobility value. When analyzing the other protein fractions of *Acanthamoeba castellanii* strains Neff and 309 one may find that those strains, though different in their pathogenecity, belong to the same species.

The analysis of proteins of *Naegleria fowleri* strains revealed that both the strains, the pathogenic strain Vitek and the nonpathogenic strain 5 D, demonstrate 100% resemblance, which confirms that they belong to the same species.

Thus, at the present stage of investigations, it may be stated that the electrophoretic analysis of proteins on polyacrylamide gel makes it possible to determine which species of the amoeba strains studied belong to, but it does not allow to differentiate pathogenic and non-pathogenic strains belonging to the same species.

The electrophoretic distribution of proteases on agar gel according to Herd and Motycka (1973) is a new technique which makes it possible to evaluate a fraction of proteolytic properties quantitatively. Interesting is the fact of finding 5 fractions of non-specific proteases in the pathogenic strain of both the species, *Acanthamoeba castellanii* and *Naegleria fowleri*, and 4 fractions in the non-pathogenic ones. The electrophoretic investigations revealed an electrophoretic resemblance of the fractions both within the species and between the genera investigated.

The agar electrophoresis applied to detecting proteolytic activities of proteins seems to be a very promising method as, in this way, it is possible not only to establish the differences in proteases between species and genera, but also to determine the quantity and activity of the proteases detected. This method, when provided with new techniques of selective staining of proteases, might also make it possible to determine the nature of the enzymes detected.

The suggestions made by some investigators (von Brand 1973, Venkateson et al. 1977) of one of the proteases, viz. trypsin, having a share in pathogenicity, gave rise to an idea of investigating it in free-living amoebae. The investigations by Jarumulinta and Maegraith (1961) did not reveal its presence in non-pathogenic strain Neff of *Acanthamoeba castellanii*. Our investigations partly confirm those results; the differences in trypsin activity are observed as related to the growth phase of protozoa. The non-pathogenic strain Neff of *Acanthamoeba castellanii* showed but trace activity of that enzyme (0.2 IU) in the logarithmic growth phase (4 days), whereas the pathogenic strain 309 of *Acanthamoeba castellanii* demonstrated about 30 times greater trypsin activity in the same growth phase (about 7 days), the trypsin activities of both the strains of *Acanthamoeba castellanii* were alike.

The trypsin activity in the pathogenic strain Vitek and non-pathogenic strain 5 D of *Naegleria fowleri* was different from that in *Acanthamoeba castellanii*; in the logarithmic growth phase, the activity level was similar in both the strains (14–16 IU). In the initial period of the stationary growth phase of the pathogenic strain the activity level rose twice as high (from 16 to 35 IU) to return to its initial volume in the further period of growth. In the non-pathogenic strain, however, the trypsin activity remained at more or less the same level throughout the culture growth.

These results suggest that the trypsin activity may serve as a determinant of pathogenicity or non-pathogenicity of *Acanthamoeba castellanii* strain, while the trypsin activity in the pathogenic strain of *Naegleria fowleri* twice as high as in the non-pathogenic strain can hardly be regarded as an unmistakable exponent of this pathogenicity.

The results of investigations on the acid and alkaline phosphatase indicate that the pathogenic strains of both *Naegleria fowleri* and *Acanthamoeba castellanii* demonstrate in the logarithmic growth phase about four times higher activity of the acid phosphatase and about twice higher activity of the alkaline phosphatase than the respective non-pathogenic strains. The activity of those enzymes drops distinctly in older (7 days) culture of pathogenic strains. The acid and alkaline phosphatase activities in the non-pathogenic strain, on the other hand, rise up to the 7th day of culture reaching the same value as those characteristic of the pathogenic strains in that period. The differences in the activity of those enzymes may be indicative of a more intensive metabolism of pathogenic strains and of a possible role of the phosphatases played in the pathogenicity. The drop in the activity of the phosphatases on the 7h day of culture in the pathogenic strains may be due to an inhibition of the culture grown in a closed system or to a depletion of the metabolic potential. Although the role of phosphatase in pathogenicity still requires investigations on a larger number of pathogenic and non-pathogenic strains of amoebae of *Acanthamoeba* and *Naegleria* species, there are already papers suggesting a possible share of lytic enzymes in this process. Culbertson et al. (1968) assume that the capability of *Naegleria fowleri* to penetrate into the host's tissue may be dependent on specialized cytolytic enzymes. Chang (1971) and Visvesvara and Calaway (1974) confirm this when finding in tissue cultures some changes of cytolytic character brought about by amoebae.

The investigations on isozymic fractions of the acid phosphatase revealed differences in the number of fractions between amoeba strain; 5 fractions were found in both the pathogenic and the non-patogenic strains of *Acanthamoeba castellanii* and 8 fractions in pathogenic strain Vitek and 5 fractions in non-pathogenic strain 5 D of *Naegleria fowleri*. The acid phosphatase occurring in protozoa is, as a rule, represented by very numerous isoenzymes (Ebert 1973, 1974 a and b).

The heterogeneity of phosphatases in the strains investigated is indicative of great specificity of these enzymes in regard to the substrates. Equally great is number of isoenzymes of non-specific esterase in both *Acanthamoeba castellanii* and *Nae-*

gleria fowleri strains. As is known, the esterases catalyze many enzymatic reactions to begin with hydrolysis of simple carboxylic esters to end with very complicated organic compounds. The great number of esterases of different electrophoretic properties in pathogenic strains of the species studied may account for the manifold role of these enzymes not excluding their share in pathogenicity of protozoa.

The histochemical investigations of acid phosphatase carried out by light microscopy technique revealed a uniform distribution of the enzyme in the cells of both pathogenic and non-pathogenic strains of either species (*Acanthamoeba castellanii* and *Naegleria fowleri*). As observed in spectrophotometric investigations, the acid phosphatase activity in non-pathogenic strains was lower than that in the pathogenic ones as it was only detected after a longer time of incubation. The histochemical investigations by light microscopy technique seem to be useful merely for quick determination of great differences in enzymatic activity of protozoa, as the difference of four times higher activity of acid phosphatase revealed biochemically in pathogenic strains remained unperceived in histochemical incubation.

The investigations on lytic enzymes carried out in this study were aimed at verifying a hypothesis of their share in free-living amoebae becoming pathogenic. The results of investigations on the activities confirm the conjecture about their possible share in this process. At this stage of studies, however, it is hardly possible to state explicitly that the role of these enzymes is decisive. Perhaps the pathogenicity is a requisite of still other enzymes not yet given due attention or else depends on a number of other factors, different metabolic features or an immunologic response of host to the presence of parasitic protozoa.

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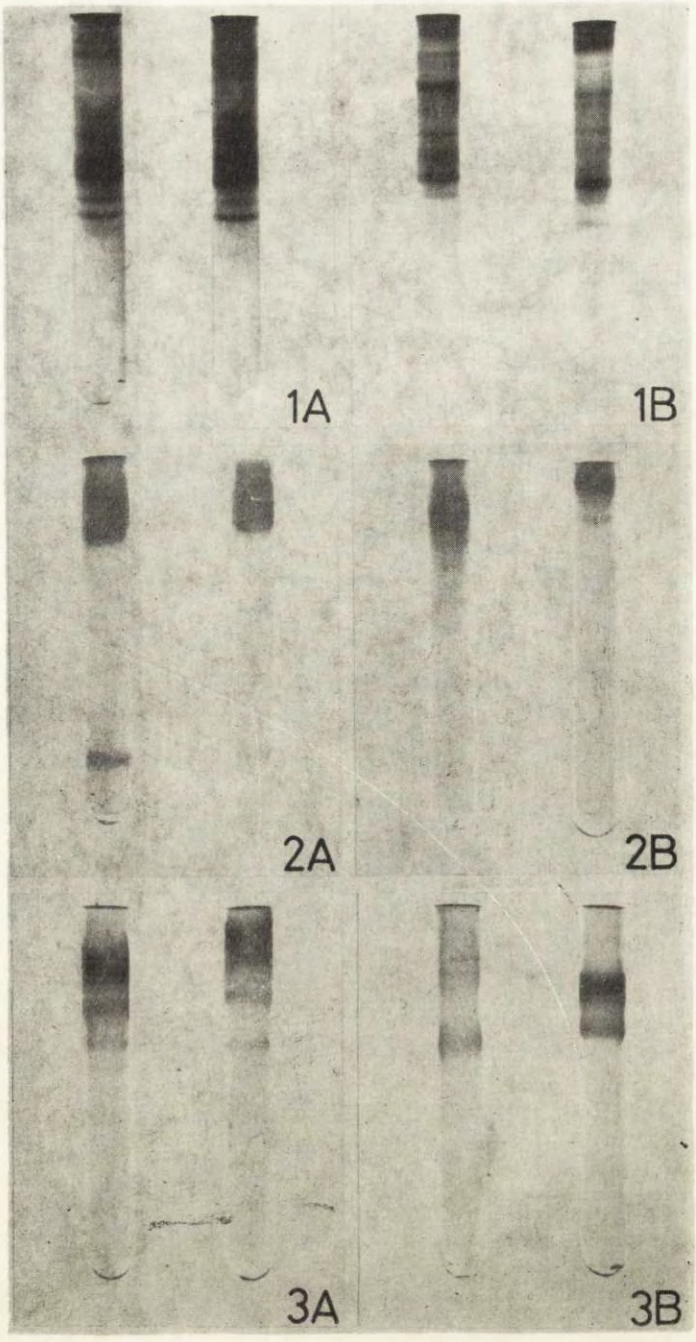
EXPLANATION OF PLATES I–III

1: Electrophoretic distribution of protein fractions on the polyacrylamide gel, A — *Acanthamoeba castellanii* strain Neff and 309, B — *Naegleria fowleri* strain Vitek and 5 D
2: Electrophoretic distribution cf acid phosphatases on the polyacrylamide gel, A — *Acanthamobea castellanii* strain Neff and 309, B — *Naegleria fowleri* strain Vitek and 5 D
3: Electrophoretic distribution of non-specific esterases on the polyacrylamide gel, A — *Acanthamoeba castellanii* strain Neff and 309, B — *Naegleria fowleri* strain Vitek and 5 D
4: Histochemical localization of acid phosphatase in the cells of *Acanthamoeba castellanii* strain 309
5: Histochemical localization of acid phosphatase in the cells of *Acanthamoeba castellanii* strain Neff
6: Histochemical localization of acid phosphatase in the cells of *Naegleria fowleri* strain Vitek
7: Histochemical localization of acid phosphatase in the cells of *Naegleria fowleri* strain 5 D
8: Histochemical localization of non-specific esterases in the cells of *Acanthamoeba castellanii* strain 309
9: Histochemical localization of non-specific esterases in the cells of *Acanthamoeba castellanii* strain Neff
10: Histochemical localization of non-specific esterases in the cells of *Naegleria fowleri* strain Vitek
11: Histochemical localization of non-specific esterases in the cells of *Naegleria fowleri* strain 5 D



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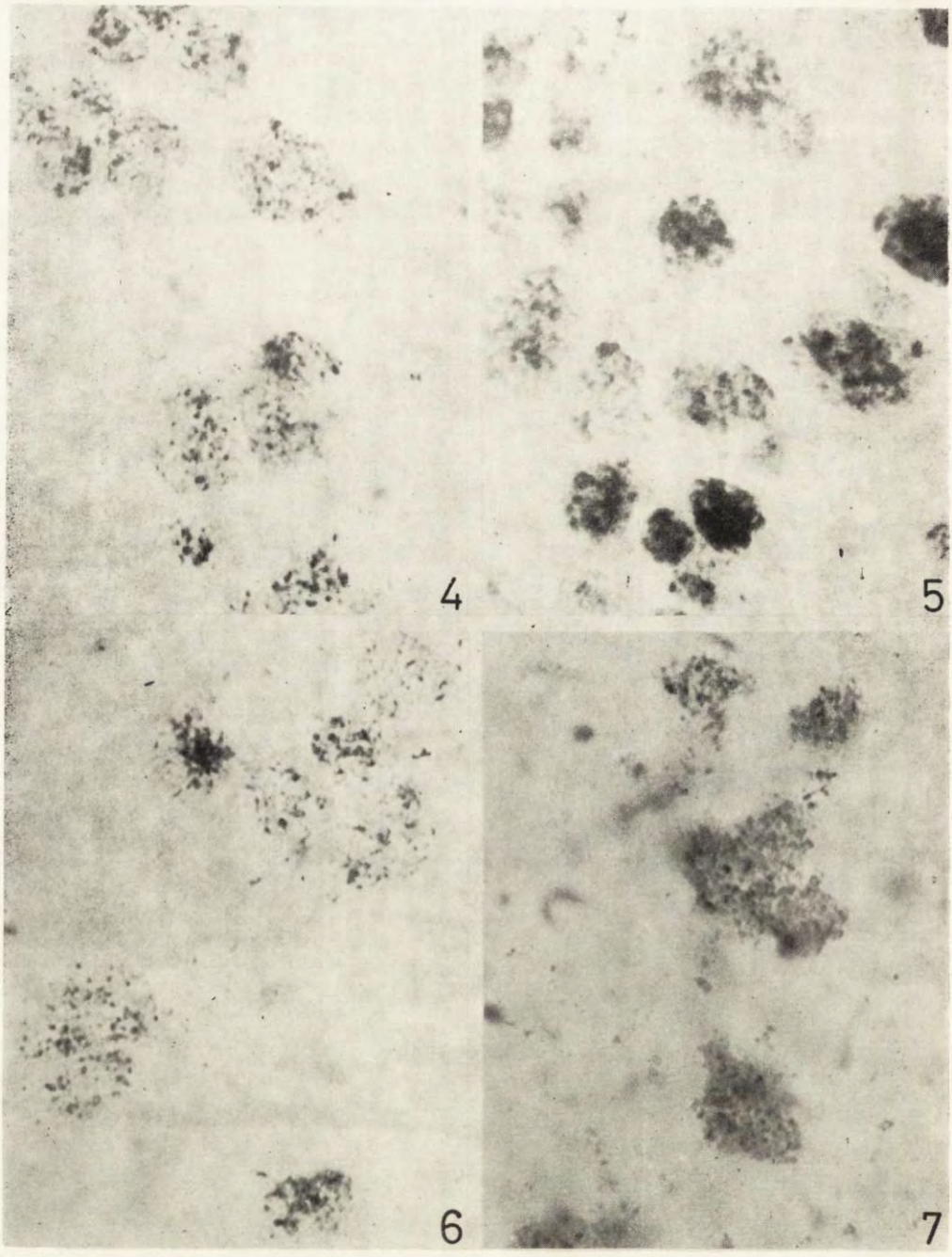

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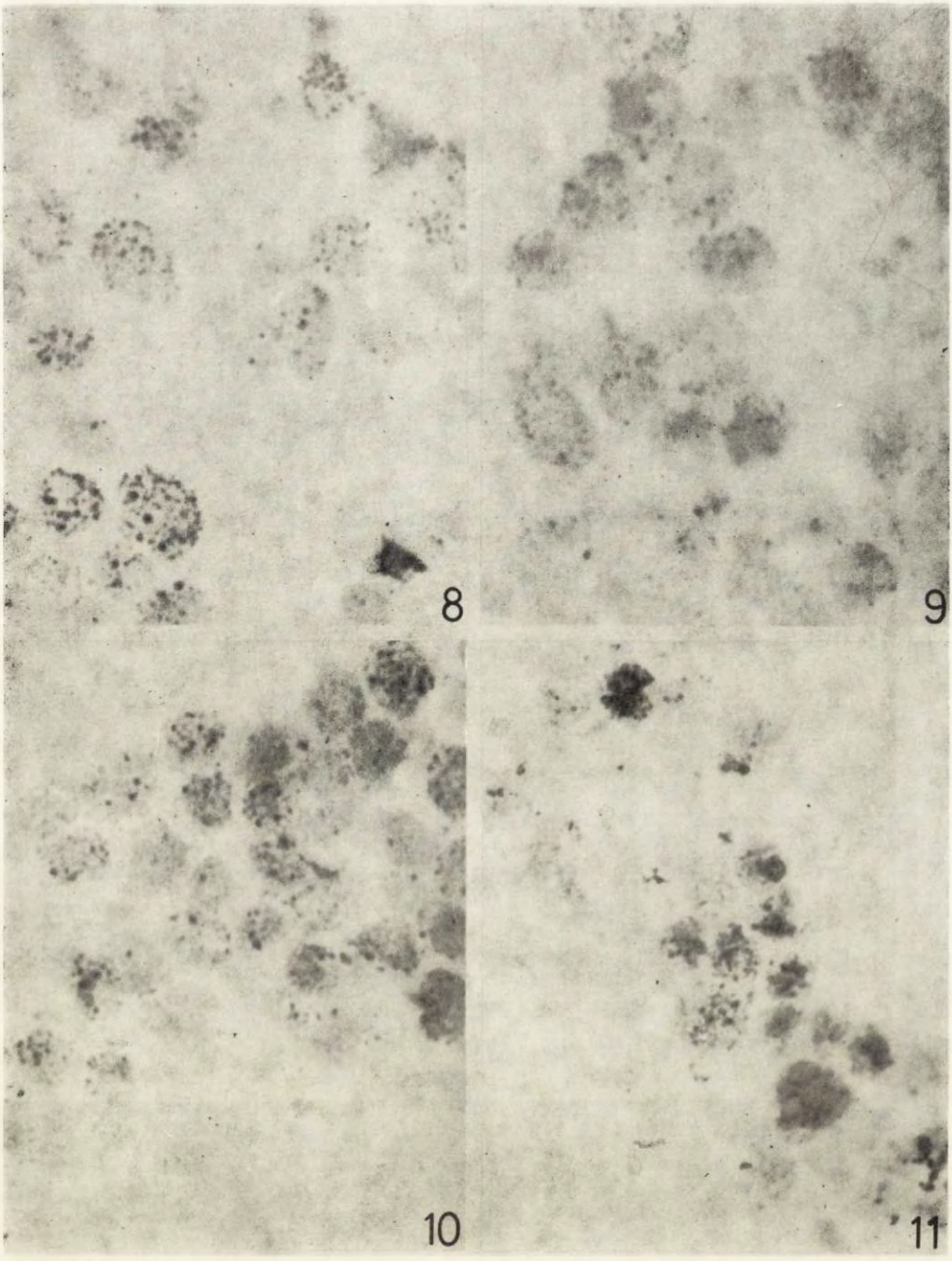

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ACTA PROTOZOOLOGICA

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Nosema euzeti sp. n. and *Gregarina euzeti* sp. n., Two New Protozoan Parasites of a Mite *Euzetes seminulum* (O. F. Müller) (*Acarina, Oribatei*)

Received on 18 July 1981

Synopsis. A new microsporidian *Nosema euzeti* sp. n. and a new eugregarine *Gregarina euzeti* sp. n. are described from larvae and adults of a mite *Euzetes seminulum* (O. F. Müller). The spores of *Nosema euzeti* sp. n. fixed and stained with Giemsa's solution are 3.1–4.3 by 1.9–2.5 μm. This microsporidian causes a general infection of its host. The sporonts of *Gregarina euzeti* sp. n. form biassociative syzygies and they inhabit the intestine. The maximum length of sporonts is 128 μm and their width 78 μm. The range of ratio length of protomerite to the total length of sporont (LP : TL) is 1:2.9–7.2 and the ratio of protomerite width to deutomerite width is 1 : 1.1–4.4. The tabulated records of microsporidian and gregarine infections of mites are given.

The mite *Euzetes seminulum* (O. F. Müller) (= *Euzetes globosus* Nicollet) is a common inhabitant of leaf litter in parks and forests in Europe but does not occur in open agricultural fields. It is a saprophagous organism feeding probably only on decaying plant material.

Material and Methods

First infected specimens of *E. seminulum* were received by Prof. Dr. Jan Rafalski, Poznań University in September of 1972. During the period 1977–1980 at the irregular intervals specimens of *E. seminulum* were collected in Rogalin where first infected specimens were found.

Mites were squashed in water on microscopic slides and their tissues and gut content were microscopically examined on the presence of parasites. Smeared tissues were fixed in absolute methanol for 2 min and stained in 0.25% Giemsa's stain for 24 h.

Results

Altogether 166 adults and larvae of *E. seminulum* were microscopically examined and results of diagnoses are given in Table 1. Two new protozoan parasites were

recorded and described as a microsporidian *Nosema euzeti* sp. n. and an eugregarine *Gregarina euzeti* sp. n.

Table 1

Parasitization of population of *Euzetes seminulum* in Rogalin by *Nosema euzeti* sp. n. and *Gregarina euzeti* sp. n.

Date of sampling	Number of dissected mites	Per cent of mites parasitized by	
		Nosema euzeti sp.n.	*Gregarina euzeti* sp.n.
5. IX. 1972	43	35	51
17. III. 1977	21	5	10
28. XII. 1979	78	31	6
14. VIII. 1980	17	18	12
23. X. 1980	7	14	71
Total	166		

Nosema euzeti sp. n.

Host: *Euzetes seminulum* (O. F. Müller)
Habitat: general infection
Locality recorded: Rogalin 5. IX 1972, 17. III. 1977, 28. XII. 1979, 14. VIII. 1980, 23. X. 1980; Sępólno Krajeńskie 25. VII. 1972.

Merogony and Sporogony

The development of this species is typical for the genus *Nosema*.

The typical meronts (schizonts) are spherical in shape having up to 7 μm in diameter (Pl. I 1). They have two relatively large nuclei stained light red. The cytoplasm around the nuclei is light blue while the outer is more dense and therefore more deeply stained. Tetranuclear schizonts were only occasionally seen.

The sporonts are diplokaryotic and have 3–4 μm in diameter. The binucleated sporoblasts are oval and measure 4–5 × 3 μm.

Spore

Spores mature individually. They are elongately oval and take Giemsa stain intensively (Pl. I 2). The sporoplasm is conical with a vacuole at the anterior pole.

Two small nuclei are seen inside the sporoplasm.

The fresh spores measured in water were 3.2–4.7 × 1.9–2.6 μm. Spores fixed and stained were slightly smaller and measured 3.1–4.3 × 1.9–2.5 μm (Table 2).

Pathology

The microsporidian infects mainly the fat body cells, but hemocytes, ovaries and other tissues are also attacked.

Table 2

Frequency distribution of the length of spores of *Nosema euzeti* sp. n.

Spore sample	Dimensionable groups (μm)			
	3.1–3.5	3.6–4.0	4.1–4.5	4.6–5.0
Fresh	9	40	47	4
Stained	17	24	9	–

Taxonomic Position

There are two records of experimental microsporidian infections among oribatid mites: *Nosema helminthorum* Moniez in *Ceratoppia bipilis* (Hermann) and *Xenillus tegeocranus* (Hermann) (Dissanaike 1958) and *Cryptosporina brachifilia* Hazard et Oldacre (1975) in *Piona* sp. (Table 3). Besides, there are known records of five other microsporidians infecting other mites than *Oribatidae*.

As it is seen in the Table 3 the microsporidian recorded in *Euzetes seminulum* greatly differs by the size of spores and tissues attacked from all microsporidians known from mites. For this reason I consider that the microsporidian found in *Euzetes seminulum* has never been recorded and described previously and for this reason I propose for it the name *Nosema euzeti* sp. n.

Table 3

Microsporidians recorded from mites (*Acarina*)

Species	Host	Tissues infected	Spore size in μm
Cryptosporina brachifilia Hazard et Oldacre, 1975	*Piona* sp.	Fat body	1.80–1.91 × 0.53–0.80
Gurleya sokolovi Issi et Lipa, 1968	*Limnochares aquaticus* (L.)	Fat body, hemocytes, nerves	6.9 × 3.5 4.5–6.6 × 1.9–3.2
Microsporidium ixodis Weiser, 1957	*Ixodes ricinus*	No data	1.6–2.6 × 1.2–1.4
Nosema euzeti sp. n.	*Euzetes seminulum* (O. F. Müller)	General infection	3.2–4.7 × 1.9–2.6 3.1–4.3 × 1.9–2.5
Nosema helminthorum Moniez, 1887	*Ceratoppia bipilis* (Hermann) *Xenillus tegeocranus* (Hermann)	Midgut and caeca	5.81–6.8 × 3.25
Nosema sperchoni Lipa, 1962	*Sperchon* sp.	Fat body	4.5–5.5 × 3.0–3.5
Nosema steinhausi Weiser, 1956	*Tyrophagus putrescentiae* (Schr.) (= *T. noxius* A.Z)	Fat body, lymphocytes	2.8 × 1.3

Gregarina euzeti sp. n.

Host: *Euzetes seminulum* (O. F. Müller)
Habitat: intestine
Locality records: Rogalin 5. IX. 1972, 17. III. 1977, 28. XII. 1979, 14. VIII. 1980, 23. X. 1980, Sępólno Krajeńskie 25.VII.1972.

Morphology

Sporonts in associations (Pl. II 6). Maximum length of sporonts 127 μm; maximum width 72 μm. Ratio LP:TL = 1 : 2.9–7.2; ratio WP : WD = 1 : 1.1–4.4 (Table 4).

Table 4

Measurements of living sporonts and trophozoites of *Gregarina euzeti* sp. n.

	TL	LP	LD	WP	WD	LP : TL	WP : WD
Sporonts							
Prim.	50	11	39	17	26	1 : 4.5	1 : 1.5
Sat.	47	16	31	14	25	1 : 2.9	1 : 1.8
Prim.	48	12	36	17	34	1 : 4	1 : 1.2
Sat.	43	9	34	16	30	1 : 4.7	1 : 1.8
Prim.	47	11	36	16	36	1 : 3.2	1 : 2.2
Sat.	47	8	39	16	34	1 : 5.8	1 : 1.8
Prim.	64	14	50	14	44	1 : 3.6	1 : 3.1
Sat.	59	11	48	23	47	1 : 5.4	1 : 2
Prim.	63	13	50	19	39	1 : 4.8	1 : 2
Sat.	56	9	47	19	37	1 : 6.2	1 : 1.9
Prim.	72	14	48	20	47	1 : 5.1	1 : 2.2
Sat.	76	14	62	19	39	1 : 5.4	1 : 2.0
Prim.	67	16	51	22	53	1 : 4.2	1 : 2.4
Sat.	79	12	67	31	62	1 : 6.6	1 : 2
Prim.	81	16	65	26	72	1 : 4.1	1 : 2.8
Sat.	73	23	56	14	62	1 : 3.1	1 : 4.4
Prim.	80	16	64	26	72	1 : 4	1 : 2.8
Sat.	80	11	69	28	62	1 : 7.2	1 : 2.2
Prim.	124	27	97	36	50	1 : 4.6	1 : 1.4
Sat.	127	23	104	34	50	1 : 5.5	1 : 1.5
Trophozoites							
	87	20	67	31	50	1 : 4.3	1 : 1.6
	92	17	75	22	25	1 : 5.4	1 : 1.1
	94	16	78	22	30	1 : 5.8	1 : 1.4
	94	28	66	31	36	1 : 3.3	1 : 1.1
	98	19	80	36	78	1 : 5.1	1 : 2.1
	111	19	92	25	33	1 : 5.8	1 : 1.3
	120	25	95	42	72	1 : 4.8	1 : 1.7
	128	27	101	42	78	1 : 4.7	1 : 1.8

Legends: Prim. – primite, Sat. – satellite, TL – total length of gregarine, LP – length of protomerite ,LD – length of deutomerite, WP – width of protomerite, WD – width of deutomerite.

Trophozoites and young sporonts have protomerite distinctly separated by constriction and septum from the deutomerite. Epicite thin, endocyte granular and moderately translucent. Nucleus located in the front part of deutomerite (Pl. II 3, III 8).

Mature sporonts and those being in associations are elliptical or oval (Pl. II 4, 6, III 8).

Protomerite of primite semicircular, longer than wide. Septum between proto- and deutomerite well seen. Deutomerite oval or round. Nucleus up to 10 μm in diameter is located in the first half of deutomerite, not well seen. Epicite very thin, endocyte granular and dark.

Protomerite of satellite flattened, two times wider than long. Deutomerite oval or round. Nucleus seen inside the granular endocyte (Pl. II 5).

In many mites gregarines with deformed bodies were observed (Pl. II 6).

Parasitism

Parasitization of *E. seminalum* population in Rogalin during 1972–1980 was high especially in autumn (Table 1).

Number of gregarines in mites greatly varied. In some mites up to 40 gregarines were observed while in others only a few were present.

Taxonomic Position

It is the first record of gregarine infection in *E. seminulum* and the morphological features of the investigated species indicate that it belongs to the genus *Gregarina.*

In the literature there are six records of gregarine infections in mites (Table 5) but only *Gregarina scutovertexi* Erhardova and *Asterophora caloglyphi* Rauchalles (Geus 1969) were properly described. In case of *Gregarina oribatarum* Nicollet (1855) this name should he considered as nomen nudum since there is no data on morphology and hosts of this gregarine. There are also no data on morphological features of *Gregarina* sp. reported by Michael (1884) from *Damaeus oblongus* and by Wellmer (1911) from *Damaeus geniculatus* C. L. Koch (= *Oribata geniculata* L.) and therefore it is not possible to make any comparison between those species with that found in *E. seminulum.*

The gregarine *Asterophora caloglyphi* Rauchalles (Geus 1969) described from *Caloglyphus moniezi* belongs to another family than *G. euzeti* sp. n.

The gregarines described by Erhardova from *Scutovertex minutus* (C. L. Koch) differ from *Gregarina euzeti* sp. n. by their morphology and size (Table 5). None of them formed associations and *G. corolla* had a crown of hooks. A second species *G. scutovertexi* has a similar range of length but ratio LP : TL amounts 1 : 10 while in case of *G. euzeti* sp. n. amounts about 1 : 5 (2.9–7.2).

Due to these differences I consider that the gregarine recorded in *Euzetes seminulum* has not been described previously and I propose for it the name *Gregaina euzeti* sp. n.

Table 5
Gregarines recorded from mites (*Acarina*)

Gregarine	Host	Size of sporont in μm	Ratio LP : TL WP : WD	Referenes
Asterophora caloglyphi Rauchalles	*Caloglyphus moniezi* Zachvatkin	116–144×32–36	1 : 4.1–4.4 1 : 1.1–1.16	Geus (1969)
Gregarina corolla Erhardova	*Scutovertex minutus* (C. L. Koch)	120–170×10–12	not given	Erhardova (1955)
Gregarina scutovertexi Erhardova	*Scutovertex minutus* (C. L. Koch)	77–136×26–36	1 : 10 1 : 2	Erhardova (1955)
Gregarina euzeti sp. n.	*Euzetes seminulum* (O. F. Müller)	43–128×25–78	1 : 2.9–7.2 1 : 1.1–4.4	Lipa (this paper)
Gregarina oribatarum Nicollet	not given	not given		Nicollet (1885)
Gregarina sp.	*Damaeus oblongus*	not given	not given	Michael (1884) Nicollet (1885)
Gregarina sp.	*Oribata geniculata*	not given	not given	Wellmer (1911
Unidentified	*Limnochares aquaticus* (L.)	not given	not given	Issi and Lipa (1968)

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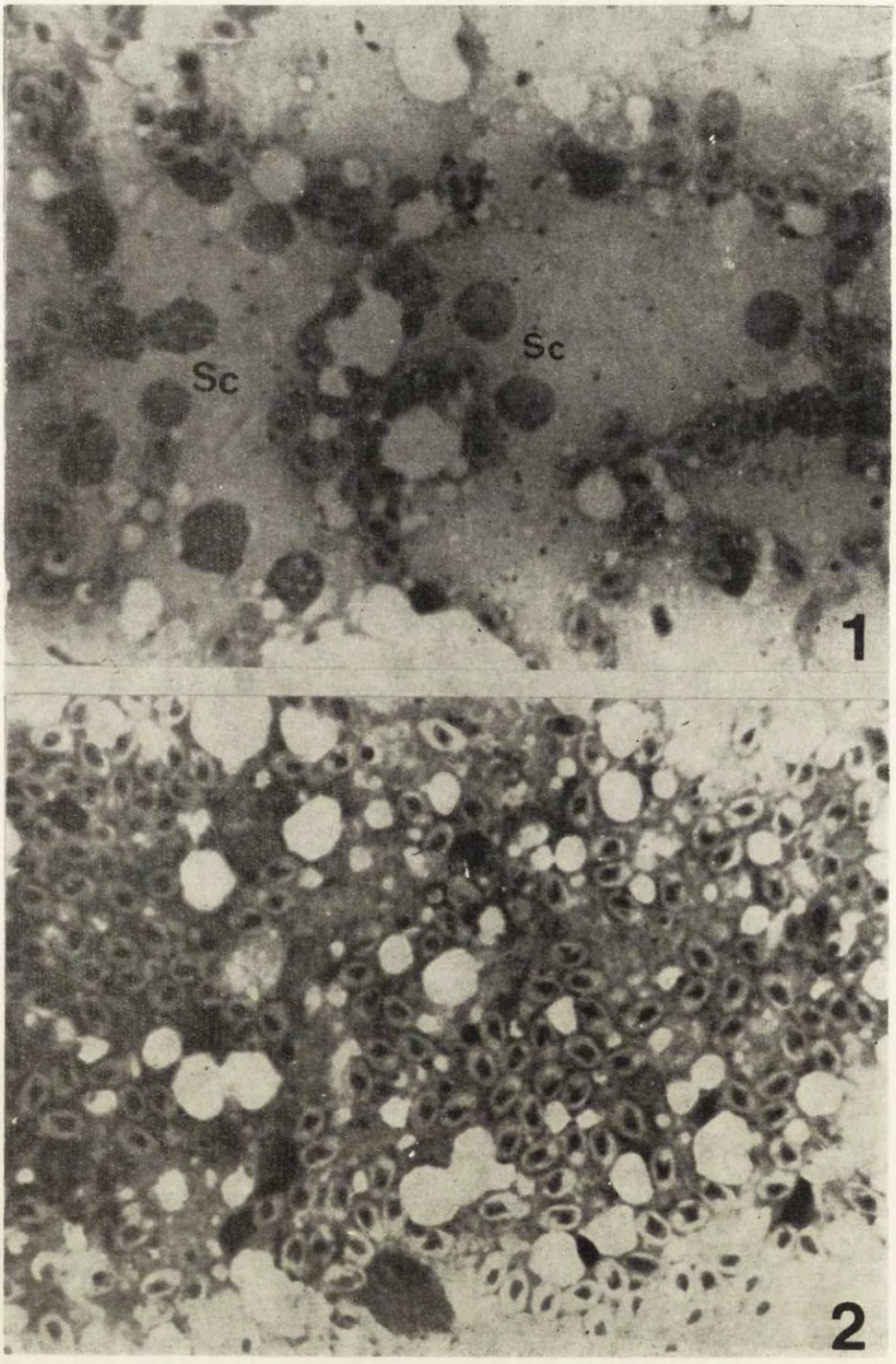
EXPLANATION OF PLATES I–III

Nosema euzeti sp.

1–2: Meronts (Sc) and spores stained with Giemsa's stain
Gregarina euzeti sp. n.
3: Typical sporont
4–5: Sporonts of different shape
6: Sporonts in association, satellite with well seen nucleus
7–8: Typical (T) and deformed (D) sporonts



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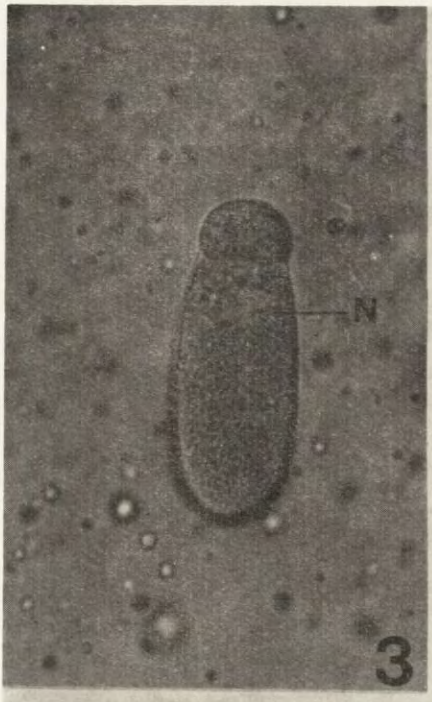

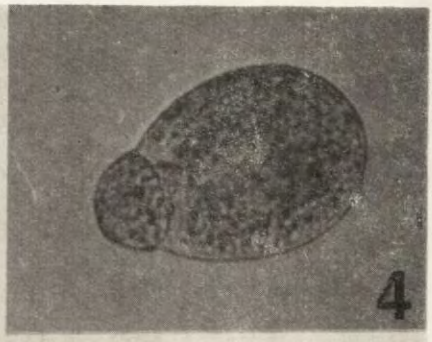

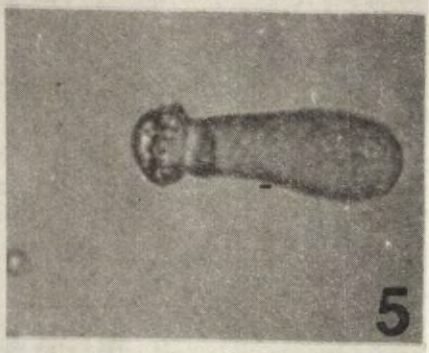

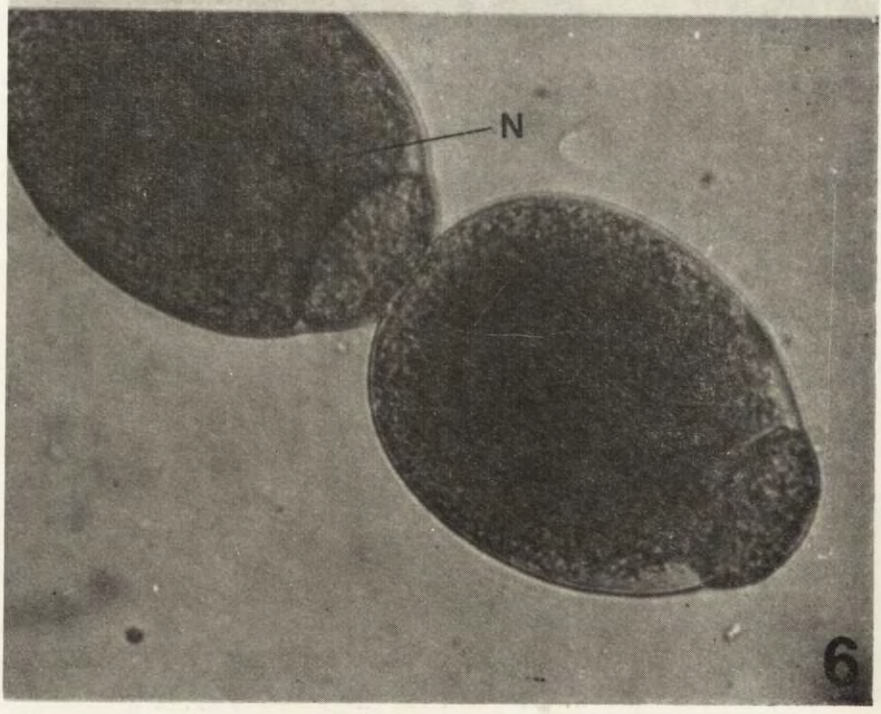

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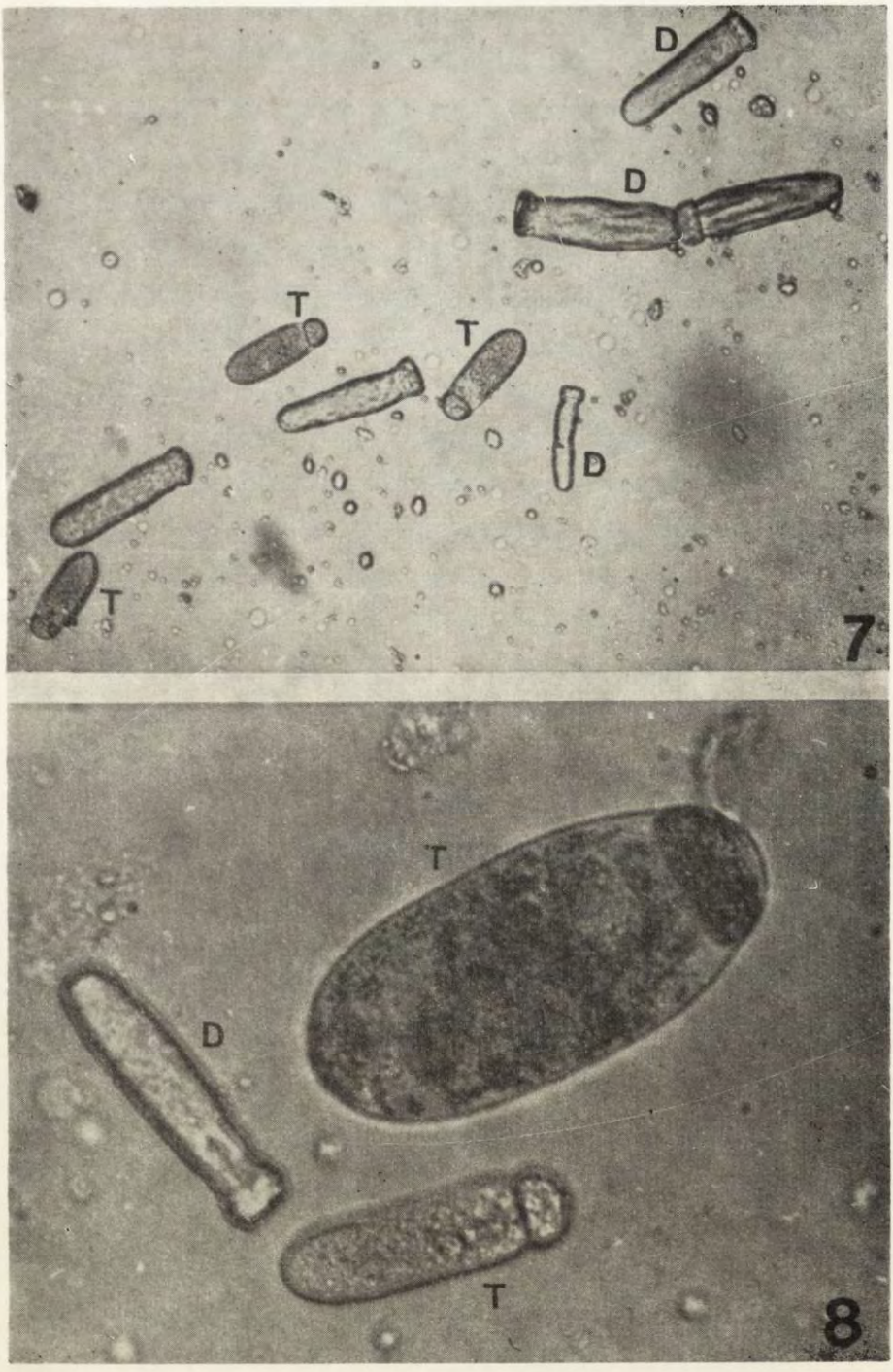

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ACTA PROTOZOOLOGICA

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A New Microsporidian, *Pleistophora atretii* sp. n. from the Fresh Water Snake, *Atretium schistosum* Günther

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Synopsis. The morphology and life-history of a new species of a microsporidian *Pleistophora atretii* sp. n. from the fresh water snake, *Atretium schistosum* Günther is described. New species is compared with three species of microsporidians: *Microsporidium danilevskyi*, *M. ghigii* and *Pleistophora heteroica* described from snakes.

Microsporidians are reported either as parasites or hyperparasites of a variety of invertebrates and vertebrates. A perusal of the literature showed that only three species of microsporidians have been reported from snakes, namely *Nosema heteroicca* Moniez, 1887, from *Zamines gemonensis* (= *Coluber carbonarius*); *Glugea danildevskyi* Pfeiffer, 1895, from *Tropidonotus natrix* as a hyperparasite of a trematode, and *Glugea ghigii* Guyenot et Naville, 1924, from *Tropidonotus natrix* as a hyperparassite of cestodes. The generic designation of *Nosema heteroica* and *Glugea danilevsskyi* caused some doubts and Labbé (1899) transferred both species to the genuss *Pleistophora*. Kudo (1924) while summarizing the literature on the microsporiddians overlooked this fact and preserved the original generic designation. Spraague (1977) in his comprehensive reviews on microsporidia lists *Pleistophora hetereoica* while he placed *Glugea danilevskyi* and *G. ghigii* in a collective group *Microsporidium* since their incomplete description does not allow to make a proper generric designation.

While examining the fresh water snake *Atretium schistosum* Günther (Fam.: *Colubbridae*, Ord.: *Ophidia*) collected in Gudivada (Andhra Pradesh, India) we came acrosss a new microsporidian parasite, *Pleistophora atretii* sp. n. which is described in thae present paper.

Material and Methods

The snakes were anaesthetized with chloroform, dissected and the viscera displaced to a side to expose the vertebral column. Infection when present was evident by the presence of opaque white cysts having a diameter of about 1.0–1.2 mm which were superficially embedded in the tissue adjacent to the vertebral column. Smears were prepared from cysts of different sizes and stained with Giemsa after an initial hydrolysis in 1 NHCl at 60°C for 10 min or stained with Heidenhain's iron haematoxylin. Smears containing the spores were also treated according to the PAS and Feulgen techniques and counterstained with light green. Cysts were fixed in alcoholic Bouin's fluid or 10% formaldehyde, sectioned at 8 μm thickness and stained with the usual histological stains like Azocarmine, Heidenhain's iron haematoxylin or Mallory's triple stain to study the morphology of the spore.

Observations

Host: *Atretium schistosum* Günther
Parasite: *Pleistophora atretii* sp. n.
Site of infection: Body muscles adjacent to the vertebral column.
Type slides: Authors' collection and also in Department of Zoology, Andhra University, Waltair, India.

Fresh water snakes belonging to the genera *Atretium* and *Tropidonotus* are commonly found in the irrigation canals in Krishna District (Andhra Pradesh, India) throughout the year and in greater numbers during the monsoon (Aug.–Oct.). A total number of 248 *Tropidonotus* and 48 *Atretium* collected during the different months of the year were examined for microsporidian parasites and 4 specimens of *Atretium* measuring about 70 cm each were infected with the parasite. There were no external indications of infection and the snakes were active. Opaque white cysts measuring 700–1250 μm in diameter and covered with a thick layer of host tissue were found adjacent to the vertebral column. The cysts were mostly packed with fully developed spores. Pansporoblasts enclosing a variable number of spores and a few earlier stages of sporogonial plasmodia were found in a thin layer of hyaline cytoplasm along the periphery of the cysts (Fig. 1 *14*). Smaller cysts ranging in diameter from 200–350 μm contained a large quantity of cytoplasm and developing sporogonial plasmodia along the periphery.

There was only one merogonial sequence. Smears prepared from smaller cysts showed irregularly shaped meronts measuring 8.5–10.6 μm (Fig. 1 *1*) and contained variable number of nuclei. A maximum number of 24 nuclei were found in a single meront. Each nucleus appeared in the form of 2 or 3 deeply stained chromatin granules and a distinct nuclear membrane was not observed. The cytoplasm was hyaline and lightly stained. Some of the larger meronts were found undergoing plasmotomy (Fig. 1 *2*) resulting in the formation of smaller and smaller plasmodia, ultimately resulting in the formation of linearly arranged uni-nucleate bodies

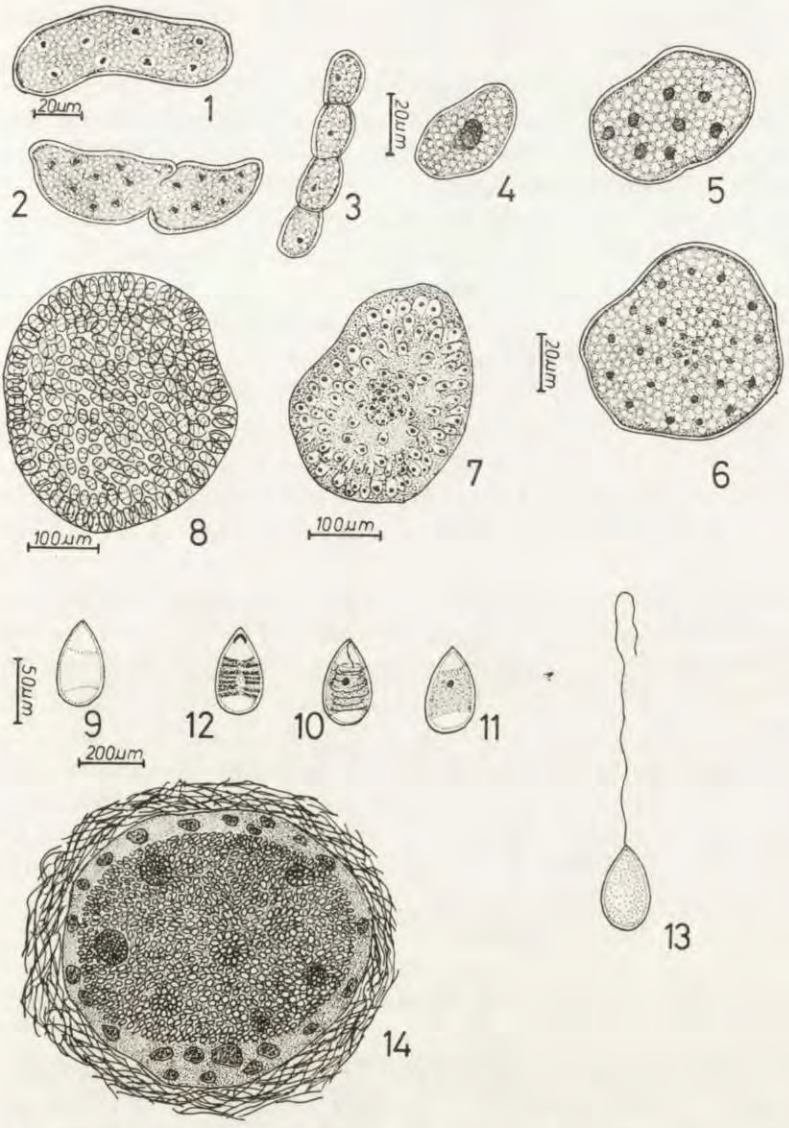

Fig. 1. *Pleistophora atretii* sp. n. *1* — A meront, *2* — Meront undergoing plasmotomy, *3* — Sporonts arranged in a linear row, *4* — A uninucleate sporont, *5–6* — Stages in sporogonial sequence, *7* — Sporogonial plasmodium: Note the developing sporoblasts along the periphery, *8* — A pansporoblast, *9* — A fresh spore, *10* — A spore stained with Giemsa, *11* — A spore treated with Feulgen, *12* — A spore treated according to PAS technique, *13* — A spore showing extruded polar filament, *14* — A section of a cyst showing developmental stages along the periphery and spores in the centre

(Fig. 1 *3*). This probably represents the initial phase in the sporogonial sequence. Uni- and bi-nucleate sporonts were observed along the periphery of the cysts. They are oval in shape measuring 4.5–5.4 × 3.8–4.2 µm containing a single deeply stained

nucleus. The chromatin material was in the form of several fine granules distributed over a wider area (Fig. 1 *4*). By repeated nuclear divisions the sporont develops into a multinucleate sporogonial plasmodia. The size of the sporogonial plasmodium and the number of nuclei contained therein increase with the growth and development of the parasite (Fig. 1 *5*). A sporogonial plasmodium measuring 10.8 × 8.2 μm showed about 24 interphase nuclei along the periphery and a few dividing nuclei in the centre (Fig. 1 *6*). The largest sporogonial plasmodium measured 28.0 × 24.0 μm and contained about 86 nuclei. The nuclei are arranged along the margin and the cytoplasm showed incipient segmentation (Fig. 1 *7*). In subsequent stages, each nucleus along with a small quantity of protoplasm transformed itself into a sporoblast. Pansporoblasts with varying number of nuclei were rarely seen in smears, but in sectioned material, pansporoblasts of different sizes, each surrounded by a thin delicate PAS positive membrane were clearly seen. The largest pansporoblast measured 32.8 μm in diameter and contained about 360 spores (Fig. 1 *8*) and the smallest one measured 8.2 μm in diameter and contained 32 spores. The spores were oval, slightly tapering anteriorly and measured 4.8–5.4 × 1.8–2.5 μm. They were refractile and surrounded by a thick rigid wall. An anterior polaroplast and a posterior vacuole were clearly seen (Fig. 1 *9*). Spores stained with Giemsa showed a single centrally located dot-like nucleus (Fig. 1 *10*). The nucleus appeared like a small pink granule in spores treated according to Feulgen's technique (Fig. 1 *11*). A "∧" shaped PAS positive polar cap is present at the anterior end (Fig. 1 *12*). The polar filament originates near the polar cap and extends postero-laterally in the form of a coiled watch spring. In Giemsa stained material the coiled polar filament could be seen clearly but those treated according to the PAS technique showed the faintly stained filament along the margins only. The polar filament which was released by the addition of a drop of saturated aqueous urea or 5% H_2O_2 was uniformly thin and measured 20–25 μm in length (Fig. 1 *13*).

Discussion

The present form is placed in the genus *Pleistophora* because sporogony results in the production of a constant but typically large number of uninucleate sporoblasts and because there is a persistant pansporoblastic membrane which is subpersistant as polysporophorous vesicle.

So far only three species of microsporidians, *Microsporidium danilevskyi* Pfeiffer, 1895, *M. ghigii* Guyenot et Naville, 1924, and *Pleistophora heteroica* Moniez, 1887, are reported from snakes. In *M. danilevskyi* each pansporoblast contains a maximum number of 100 spores and the spores are oval or pyriform measuring 3–4 μm in length (width not given) and the polar filament is 50–70 μm long. In the case of *M. ghigii* there is no data on the vegetative stages but its spores measure 2.0 × 2.5 μm. The authors themselves were in doubt as to whether the parasite belongs

to the genus *Nosema* or *Pleistophora*. In the case of *P. heteroica* the spores are oval measuring 6–7 × 2–3 μm with a clear terminal vacuole. The pansporoblasts measure 12–18 μm in diameter and form 8, 16, 64 or more spores. No information is available regarding the vegetative stages of either of the species.

In the present form the spore is oval measuring 4.8–5.4 × 1.8–2.5 μm and the polar filament when fully extruded measures 20–25 μm in length. The pansporoblasts vary in size from 18–32 μm and contain a maximum number of 360 spores. Hence the present form differs from those already described. Further, the present form is from a new host, *Atretium schistosum* and is apparently host specific because none of the 280 *Tropidonotus* collected from the same locality showed infection. In view of what is stated the present form is considered new to science and the name *Pleistophora atretii* sp. n. after the host is proposed.

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PROGRESS in PROTOZOOLOGY
Abstracts of papers submitted to VI International Congress of Protozoology
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THE SOIL PROTOZA

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"Nauka", Leningrad 1980

CONTENTS

Muzafarov A. M., Mavlyanova M. I., Nasyrova Z. A. — Victor Fedorovich Nikolyuk, Nikolyuk V. F. — 60 years of research into soil protozoology in the USSR and the future projects, Geltzer Yu. G., Korganova G. A. — Adaptation of protozoa to soil conditions and the irindicatory role, Nikolyuk V. F., Mavlyanova M. I., Nasyrova Z. A. — Biologically active substances of soil protozoa and plants, Geltzer Yu. G., Gordejeva L. M., Kallinikova V. D., Laskauskaite D. A., Tapil'skaya N. V. — Soil *Euamoebidea*, Geltzer Yu. G., Korganova G A., Yakovlev A. S., Alexejev D. A. — Soil *Testacida*, Geltzer Yu. G., Geptner V. A. — The effect of pesticids on soil protozoa, Geltzer Yu. G. — Methods of studying soil protozoa.

Proceedings of the Zoological Institute, Academy of Sciences of the USSR, vol. 94

PRINCIPLES OF THE CONSTRUCTION OF THE MACROSYSTEM OF THE UNICELLULAR ANIMALS
Edited by M. V. Krylov and J. I. Starobogatov

CONTENTS

Seravin L. N. — Macrosystem of the *Mastigophora*, Schulman S. S., Reschetnyak V, V. — About possibility of the superclass *Actinipoda* existence in the system of *Protozoa*, Mikhalevich V. I. — Systematics and evolution of the *Foraminifera* in view of the new data on their cytology and ultrastructure — Krylov M. V., Dobrovolsky A. A. — Macrosystem and phylogeny of the *Sporozoa*, Issi I. V. — On the present systematics of the *Microsporidia*, Schulman S, S. Podlipaev S. A. — Position of the *Cnidosporidia* in the system of *Protozoa*, Jankowski A. W. — Conspectous of a new system of the phylum *Ciliophora*, Krylov M. V., Dobrovolsky A. A., Issi I. V., Mikhalevich V. I., Podlipaev S. A., Reschetnyak V. V., Seravin L. N., Starobogatov Ya. I., Schulman S. S., Jankowski A. W. — New conceptions of the system of unicellular animals

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CONTENTS

Chapter 1 — Heteromorphism of the nuclei of vegetative cells in ciliates, *Chapter* 2 — Heteromorphism of the nuclei during the sexual process in ciliates, *Chapter* 3 — Symbiotic bacteria in the nuclear apparatus of ciliates, *Chapter* 4 — Appearance of nuclear heteromorphism in other unicellular organisms

M. V. Krylov

PIROPLASMIDS (FAUNA, SYSTEMATICS, EVOLUTION)
"Nauka", Leningrad 1981, pp. 229

Springer Series in Microbiology
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BACTERIAL AND BACTERIOPHAGE GENETICS. AN INTRODUCTION
Berlin–Heidelberg–New York, Springer Verlag, 1981, 359 pp., 111 figs

CONTENTS

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ERRATUM

In the paper of Maria Wolska entitled: „Studies on the Genus *Triadinium* Fior. (*Ciliata, Entodiniomorphida*) Comparison of *Triadinium galea* Gass. and *Triadinium caudatum* Fior." issued in Vol. 20, No. 4, 1981, pp. 357-365 of Acta Protozoologica — List of References was omitted. We publish them below:

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In preparation:

S. L. Kazubski: Studies on Interpopulational Variation in Trichodinas (*Ciliata*) — R. Chiovetti, Jr. and E. C. Bovee: Initiation of Encystment by Multiple Contacts among Starving Amebas, *Naegleria gruberi* (Schardinger, 1899) — E. Perkowska: Growth of Dense Populations of *Acanthamoeba castelanii* in Small Volumes of Nutrient and the Influence of DAPI (a DNA Ligand) on Such Populations — L. Szablewski: The Effect of Penicillin G (Sodium Salt) on *Tetrahymena pyriformis* — D. M. Saxena, Rup Lal and B. V. Prasad Reddy: DDT Uptake and Metabolism in *Blepharisma intermedium* — Ndu Umeche: The Distribution of *Naegleria* spp. by Strata in Michigan Soils — H. Tamar: On the Distribution of *Halteria bifurcata* — S. Bandyopadhyay, H. Ray and B. Dasgupta: A New Species of *Trypanosoma* from an Indian Insectivorous Bat, *Rhinoma hardwickei*, Gray — N. K. Sarkar: Myxosporidan *Myxidium haldari* sp. n. (*Myxoza*: *Myxidiidae*) from Indian Tree Frog *Hyla arborea*

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CONTENTS

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Wrocławska Drukarnia Naukowa

Index 35133