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Endocytosis and Exocytosis of Doublets of *Paramecium tetraurelia*

Received on 14 November 1980

Synopsis. Three morphologically different classes of *Paramecium tetraurelia* cells, namely: symmetrical doublets, asymmetrical doublets (in recovery to a single cellular state of organization) and control singlet cells were tested for their ability to form food vacuoles. Symmetrical doublets manifested nearly doubled rate of food vacuole formation in short lasting experiments. In symmetrical doublets both their oral apparatuses acted independently at nearly control level. On the other hand the rate of defaecation, measured in the rate of releasing of labelled food vacuoles was slowed down in doublets. *In vivo* observations revealed that certain food vacuoles were transiently immobilized within the doublets cytoplasm. They are eventually egested through the one or through the both cytoprocts (cell anuses).

It is suggested that doublets do not reach complete integration of the two cytoplasmic compartments belonging to autonomous cortical components of the feeding organelles and of the cytoprocts. An imperfect territorial integration within the doublets cytoplasm may bring out a meaningful delay of turn-over of ingested food vacuoles.

Doublet, or homopolar twin cell of *Paramecium tetraurelia* contains two complete sets of cortical structures, including the feeding organelles and the cytoprocts (sites of exocytosis). It can be formed through the incomplete separation and subsequent fusion of a conjugating pair (Sonneborn 1942, Hanson 1962, Butzel 1973). This doublet divides by binary fissions reproducing its doublet condition. Doublets of *P. tetraurelia* contain either a single macronucleus which is substantially larger than that in a singlet cell, or two smaller macronuclei (Sonneborn 1963, Butzel 1973, Morton and Berger 1978). Doublets have approximately twice the DNA content and twice the

total protein content of singlets (Morton and Berger 1978). However, the cell cycle of doublets is 164% as long as that of singlets (Morton and Berger 1978).

The doublet system, though metastable, is transformed through many generations back to the single cellular state of organization. During this transformation the loss of one oral apparatus and of one cytoproct occur, but both these events may be experimentally uncoupled (Sonneborn 1963, Sibley 1974). Hence during transformation of doublets into singlets some intermediate generations of cells are produced. The advanced in simplification forms reveal pronounced asymmetry of the displacement of the two oral apparatuses on the perimeter of doublets; thus both oral apparatuses are irregularly positioned at various angles to each other on the shorter arc of the perimeter. The left one may eventually disappear to complete the process of reversion.

In this paper an attention is focussed on the rate of food vacuole formation in the regular, symmetrical doublets, in the asymmetrical doublets with two oral apparatuses, and two cytoproct and in the control singlet cells.

The question is to what extent the separate and autonomous sets of feeding organelles are able to function independently, and to what extent the rate of endocytosis is monitored for the whole entity of doublets.

In singlet of *Paramecium* the oral apparatus is the site where cell makes and releases the membrane bound food vacuoles (phagosomes) to the interior of the cell one at a time (Mast 1947, Jurand 1961, Schneider 1964). By allowing the cell to take up labelling particles it is possible to observe the rate of food vacuole formation and subsequently the rate of their release in individual cell under non-destructive conditions.

The rate of exocytosis was much less studied in *Paramecium* (Smith-Sonneborn and Rodermal 1976). In our study the rate of exocytosis is compared in cells posessing regular symmetrical disposition of cortical organelles and in the control singlets.

Kuźnicki et al. (1972) showed that in singlets of *P. tetraurelia* the cell cyclosis proceeds on certain permanent trail. Jerka-Dziadosz (1977) in study on *Paraurostyla weissei* doublets found that after one oral apparatus impairment the whole central area of the operated doublet is filled with the food vacuoles. It suggests that the cytoplasmic content in doublets is integrated while the cortical autonomy of two components is still manifested. In our study *in vivo* observations were made on the deployment of food vacuoles inside

the *Paramecium* doublets to test the level of the cytoplasmic integrity within the doublets.

Data reported here are briefly discussed in terms of the control of the endocytosis/exocytosis cycle in doublets of *Paramecium*.

Material and Methods

Paramecium tetraurelia strain 51 S were cultured in baked lettuce medium inoculated with *Klebsiella pneumoniae* and alkalized with $Ca(OH)_2$ (Sonneborn 1950) buffered with 1 mM tris (HCl) to pH 72–7.4 (Brutkowska 1963, 1967).

By mating cells of mating type O and E the conjugants were received. When tight pairs formed, they were isolated and transfered from room temperature (of about 22°C) to 35°C. While most of exconjugants separated one pair remained fused next day. This doublet provided progeny of the symmetrical doublets. Doublets maintained their regular symmetry during several dozens of generations. They were kept at a room temperatures (22°±3) and fed only two times per week to maintain the slow rate of multiplication. After about four months some cells began to manifest some slight asymmetry of oral apparatuses disposition. Following the induced autogamy in mass culture (Sonneborn 1970, 1974), two sublines were started. These sublines B and D are expected to be of the same clonal age since the next rund of autogamy appeared simultaneously after one month. All presented here experiments were made during the middle period of the clonal cycle (Smith-Sonneborn and Rodermal 1976). Mean generation time of doublets was about one fission every second day and about one fission per day in singlets.

Cells from B and D lines were periodically assayed for the appearance of autogamy (Dippell's test of autogamy 1955) and for their morphology based on analysis of silvered specimes (Frankel and Heckmann 1968). In line B the symmetrical doublets predominated (in 61 tested specimens only 8% revealed slight asymmetry of oral apparatuses disposition). In line D there was 38.5% of the asymmetrical doublets and 61.5% of derived singlets with no case of the symmetrical doublet among 49 tested cells. During following month the number of the asymmetrical doublets in B line increased to 60.6% while the number of symmetrical doublets dropped down to 39.4% (n = 330 tested specimens). Therefore only cells from B line tested during first week are reported here as a class of the symmetrical doublets. The percentage of the asymmetrical doublets in D line decreased during one month to 27.8%, while the fraction of singlets increased to 72.8% (n = 264 tested specimens). Hence D line represented a mixture of the asymmetrical doublets and singlets and these classes were easily *in vivo* discernible under dissection microscope.

For experiment the cells of the same clonal age provided from samples of roughly equal density, and kept and fed with the same culture medium, were used. Cells from B line (of the symmetrical doublets), from D line (the asymmetrical doublets) and 51 S control singlets were isolated and starved during 30 min in an adaptation medium following Kung et al. (1975) formula. Next, these cells were introduced for 6 min into a culture medium containing latex

beads (PLP 0.76 μm diameter Serva Comp.) in a final concentration of about 10^5 beads per ml. The cells were next fixed and the number of food vacuoles per cell was counted after Preer's method (1975). In experiments on the rate of endocytosis in the cells of the same cell-cycle ages, the dividing cells from the same initial samples were isolated into a fresh medium. After 4 h post-dividers were reisolated into an adaptation medium to be substarved and next tested with latex beads as described above.

The rate of release of the uptaken labelled food vacuoles was tested in cells from the same lines. Cells labelled with PLP beads during 6 min were twicely washed with a culture medium and isolated to a culture medium again. The number of remaining food vacuoles per cell was counted after elapse of 5, 15, 20, 30, 40, 50, 60, 70, 80 and 90 min. Every experiment was repeated in three independent replicas.

In vivo observations were performed on the roughly symmetrical doublets (from B line) fed with PLP particles following substarvation. After feeding the cells were either directly mounted into a Rotocompression chamber (Amer. Biol. Comp.) for further observations in the same medium, or they were replaced into a plain culture medium for 50 min and next reisolated into a Rotocompression chamber. The latter cells posessed only 2–5 labelled food vacuoles which are readily observable in slowly swimming cells. During these observations cells were able to swim and to rotate being very slightly flattened.

Results

(1) The Rate of Food Vacuoles Formation in the Symmetrical Doublets, the Asymmetrical Doublets and in the Singlets

Results of the experiments performed on substarved cells of the variable cell ages are summarized in a Table 1.

From this Table is evident that the total number of food vacuoles formed during first 6 min of feeding in the symmetrical doublets is nearly twice (1.84) as in the controls. These values are roughly uniform

Table 1

Mean number of food vacuoles per cell formed during 6 min of feeding at 22°C in specified classes of specimens of *Paramecium tetraurelia*
n = total number of tested cells of a given class

No. of experiments	n	Regular doublets mean number and sd	n	Asymmetrical doublets mean and sd	n	Singlets mean number and sd
I	80	11.23±2.60	53	7.56±2.05	76	6.80±1.47
II	60	10.96±2.49	49	6.49±1.98	91	5.42±1.64
III	85	10.02±2.11	34	7.80±0.85	85	6.24±0.90
Total	225	10.77±1.70	136	7.27±1.68	252	6.05±1.36

for specimens of both morphological classes (low sd values) and are statistically different at a level of probability 0.05.

The class of the asymmetrical doublets presents the intermediary mean values of the ingested food vacuoles per cell and these values are not statistically different either from those of singlets, or from those of the symmetrical doublets.

Comparison of the rate of food vacuoles formation in the symmetrical doublets and in the singlets of the same cell-cycle ages revealed that during 6 min there is formed about 11.5 ± 0.9 ($n = 50$) food vacuoles per cell in the symmetrical doublets, whereas in the singlets the total number of food vacuoles averages of 6.5 ± 1.5 ($n = 97$). Cochran and Cox test again revealed that both these values are different ($p = 0.05$).

(2) The Rate of Exocytosis of the Labelled Food Vacuoles Ingested during 6 min by the Symmetrical Doublets and by the Singlets

Results of these experiments are presented in Fig. 1. From examination of curves plotted in Fig. 1 is evident that the release of food vacuoles up to 50 min proceeds parallely and roughly linearly in both classes of specimens. It indicates that the number of food vacuoles egested during the same unit of time in doublets and in singlets is roughly equal during the first 50 min of experiments. However, while the total release of food vacuoles in singlets was achieved

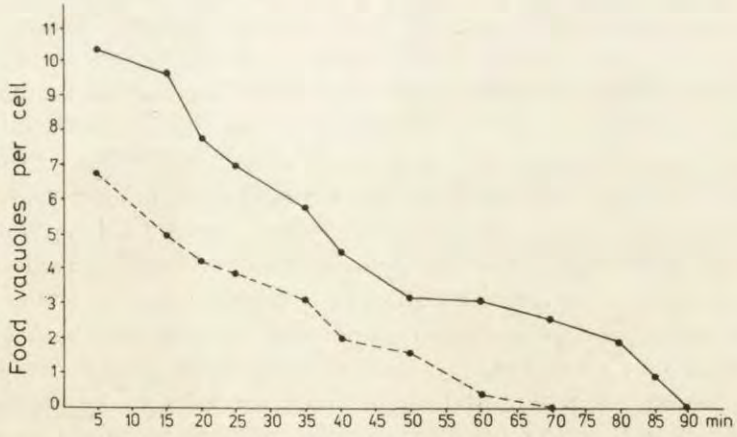

Fig. 1. Mean number of food vacuoles persisting in *Paramecium tetraurelia* cells after elapse of time indicated on abscissa. Upper curve the rate of exocytosis of the symmetrical doublets, lower curve the rate of exocytosis in the control singlets. The number of labelled food vacuoles counted in individual cells after 6 min feeding with PLP particles. Each point corresponds to a mean value of counting of at least 30 specimens. Standard deviation bars omitted for the clarity of drawing. Sd during first 50 min of experiments averages in limits ± 1.7. Sd in doublets tested after 50 min and more revealed high sd values ± 2.8

after 60–70 min, certain number of food vacuoles were still maintained in doublets to be eventually released after about 80–90 min from the start of experiments. While during first 50 min of experiments cells revealed the uniform release of food vacuoles (low sd values), the doublets during a latter period (50–90 min after the start of experiments) manifested the pronounced variability of the number of retained food vacuoles (marked as the plateau of the upper curve at Fig. 1). These vacuoles were irregularly defaecated. The whole process of the egestion was completed in particular doublet cells within limits of about 20 min i.e., from 70 to 90 min from the beginning of experiments.

(3) *In vivo* Observations on the Feeding and the Defaecation of Food Vacuoles in Symmetrical Doublets

In slightly flattened individuals the formation and movements of food vacuoles was observed in doublets in the presence of PLP beads. The both oral apparatuses were able to cumulate beads particles in the peristomal funnel, to form food vacuoles and to release them into the posterior compartment of the cytoplasm. Thus the first descending loops of the food vacuoles trails were completely independent to each other. After about 10 min of feeding the central portion of a doublet cytoplasm is filled with food vacuoles spreaded around two macronuclei. At this time it is not possible to discern the separatness of groups of food vacuoles provided by the individual oral apparatuses.

Cells chased after 50 min from 6 min lasting feeding with the PLP particles posessed only few (2–5) food vacuoles per cell. These remnant food vacuoles were distributed irregularly inside the cytoplasm. They were observed immobilized at the same locations during further minutes. They adhered to the cytoplasm close to the macronuclei, predominantly in the anterior part of the cell. Occasionally these food vacuoles moved posteriorly and then were quickly cumulated close to the one or both cytoproct. In one observation, three food vacuoles were defaecated portioned into two cytoproct. In other specimens, the asynchronous defaecation through two cytoproct was observed.

If the effect of the compression of cells on the functioning of both cytoproct is not excluded, the only reliable observations are that two separate regions of a cumulation of food vacuoles to be egested exist in doublet, and that the both cytoproct are able to function.

Discussion

From data reported here is evident that in the substarved symmetrical doublets both oral apparatuses are able to form food vacuoles.

The rate of food vacuoles formation in these cells is nearly doubled as compared to that rate of the singlets. These facts are consistent with conclusion of the physiological autonomy of both oral apparatuses.

On the other hand, the asymmetrical doublets manifested lowered rate of food vacuoles uptake. It follows from the above that during morphological reversion to the single state of cellular organization the rate of the uptake of food vacuoles concomitantly decreased. It is worth to notice that these cells yet manifested a perfect morphology of both oral apparatuses. Thus either the asymmetry of location of both oral apparatuses or the internal control of the ability to form the food vacuoles is impaired in these cells.

The rate of the exocytosis in the symmetrical doublets is comparable to the rate of an egestion performed by singlets. Since both cytoprocts are functional the slow rate of defaecation probably involves both cytoprocts. *In vivo* observations seem to indicate that the first and last portions of the cyclotic paths are independent in doublet for particular set of the ingestion/egestion set of the cortical organelles. However, the movements of food vacuoles inside the cytoplasm are crippled, and did not reach the control speed characteristic for singlets. Some food vacuoles seemed to be temporarily immobilized at random locations, in both kinds of cells having one or two macronuclei. Trapped food vacuoles are occasionally freed out into one of the cyclotic paths leading to cytoprocts and then they are finally released. These kinds of observations suggest that the structural integration of the internal organization within the doublet's cytoplasm is not reached. Thus this interpretation suggests the mechanical rather than physiological origin of the slow turn over of food vacuoles in doublets.

Allen (1974), Allen and Wolf (1974) and Allen and Fok (1980) proved that within the *Paramecium* single cells the recycling of the membrane material from defaecated food vacuoles takes place to restore the pool of the disk-bodies vesicles used for the new food vacuoles formation. In substarved doublets evidently this pool is sufficient to form during following 6 min nearly doubled number of food vacuoles. The slow turn-over of food vacuoles inside the doublets probably based on the mechanical impairment of the cyclosis may, however, bring out the secondary physiological effect on the endocytosis/exocytosis cycle. The slow rate of defaecation is followed by delay of the recycling of disk-bodies necessary for the sequent food vacuoles formation. Hence after substarvation the symmetrical doublets are able to form food vacuoles at the normal level of the efficiency of each of the oral apparatuses. However, when this pool is exhausted doublets must wait longer time for the reconstitution of this pool than singlets.

The slow input of these disk-bodies impaires reactivation of the oral apparatuses activity in doublets. Thus we suggest that feed back mechanism of the recycling of disk-bodies induces the slow rate of the turn-over of food vacuoles. In effect, it slows down the physiological machinery of the doublet. About 1.5 slower rate of the ingestion/egestion cycle in doublets as compared to singlets well fits to 1.64 slower cell cycle of doublets as compared to singlets reported in fed cells of *Paramecium* (Morton and Berger 1978).

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Excystment Cortical Morphogenesis and Nuclear Processes during Encystment and Excystment in *Laurentiella acuminata* (*Hypotrichida, Oxytrichidae*)

Received on 28 October 1980

Synopsis. Encystment and excystment in *Laurentiella acuminata* have been studied. During encystment both macronuclear fragmentation followed by fusion and micronuclear degeneration occur. The first morphogenetical event in excystment is the appearance of a few kinetosome rows. In excystment some micronuclei divide mitotically and the macronuclear mass undergoes several fragmentations without DNA synthesis.

The study of the morphogenetic and nuclear events involved in the process of encystment–excystment in ciliates has received scarce attention, and very little information about such a process in *Hypotrichous* ciliates is available at present. As far as we know, the only hypotrichous ciliate extensively studied on this line is *Oxytricha fallax* (Grimes 1973, Hashimoto 1962, 1963). Excystment and some events of the encystment have been also reported in doublets of *Onychodromus acuminatus* (= *Laurentiella acuminata*) (Jareño 1977, Jareño et Tuffrau 1979).

Both encystment and excystment involve cell differentiation processes and their study offers a great biological interest.

In the present paper the encystment–excystment cycle of the hypotrichous ciliate *Laurentiella acuminata* is reported, the attention being focussed on the morphogenetic and nuclear events.

Material and Methods

Laurentiella acuminata (Fedriani et al. 1976), a hypotrichous ciliate, was isolated from a sample of water collected at "Parque de Maria Luisa" (Sevilla).

Cultures were maintained at 20 ± 1°C in Pringsheim's medium and fed on *Chlorogonium* sp. Observations of the external organelles were made on cells stained with silver proteinate (Protargol) (Tuffrau 1967). Nuclear apparatus was stained using the Feulgen procedure. DNA contents of macro- and micronuclei were determined by microdensitometry on Feulgen stained slides, as previously reported (Torres et al. 1979).

Results

Morphology of the resting cyst. The resting cyst of *Laurentiella acuminata* presents a spherical form of 84.9 ± 0.77 μm (n = 123) in diameter. The cyst volume is approximately three times lower than that found for the vegetative cell. The outmost layer (ectocyst) of the cyst wall is formed by several plates with sinuous outline, each of them has a protuberance that gives a spiny appearance to the surface (Pl. I 1). The cytoplasm is homogeneous showing no ciliature nor infraciliature upon staining with silver proteinate (Pl. I 2).

The cyst contains only one macronuclear body disc- or band-shaped (Pl. I 3, 4) and several micronuclei which are morphologicaly similar to the micronuclei in the vegetative cells (Pl. I 4).

According to our observations on 180 resting cysts, the number of micronuclei per cyst is 3.61 ± 0.12. This figure is lower than that (4.8 ± 0.28) found in 115 cells in G_1 phase. This difference is significative at $p \leqslant 0.01$ ($t = 3.80$).

Encystment. In cultures of *Laurentiella acuminata* encystment occurs when food becomes a limitant factor. In order to induce such a process, cells were transferred a maintenance culture to fresh Pringsheim's medium without adding *Chlorogonium*. Encystment occurs eventually in most of the transferred cells, however, such a response has proved to depend on temperature (Table 1). As it can be appreciated, encystment is faster at 37°C than at 20°C. On the other hand,

Table 1

Effect of temperature on the encystment

Temperature (C°)	% Encystment		
	6 h	8 h	10 h
20	10	10	10
37	70	80	80
45	0	0	0
4	0	0	0

at temperature of about 4°C and 45°C the vegetative cells failed to encyst and eventually died.

During encystment the cell becomes rounded and its volume undergoes a decrease of about 70%. The first microscopical sign of the encystment is the resorption of the posterior tract of the endoral membrane (Pl. I 5). Thereafter, the simultaneous disappearance of the rest of cortical organelles can be observed, i.e., adoral zone of membranelles (AZM), frontoventral (FVC), transversal (TC) and marginal (MC) cirri (Fig. 2 B).

The front part of the AZM is the last organelle observable (Pl. I 6). Encystment is completed with the formation of the cyst wall (Fig. 2 D).

Encystment involves changes in the number of macronuclear fragments. Indeed, in precystic cells occur a macronuclear fragmentation (Fig. 2 C), the mean of macronuclear fragments found in 113 G_1 cells was 4.48 ± 0.07, while it was 6.71 ± 0.15 in 85 precystic cells, these values are significantly different ($t = 12.89$). The fragments so formed fuse again and form the only one cystic macronuclear mass (Fig. 2 D).

Excystment. According to our "in vivo" microscopical observations the first sign of excystment in *L. acuminata* is the restoration of the activity of contractile vacuole, probably as a result of rehydration of the cyst cytoplasm. Later on, the animal begins to rotate within the cyst wall and eventually the emergence takes place, the animal leave slowly through a wide gap in the wall (Pl. II 7, Fig. 2 F). At this moment the animal remains still enclosed by a thin envelope wich breaks soon, releasing the new vegetative cell (Fig. 2 G).

The morphogenetical processes occur before emergence takes place. Preparations stained with protargol showed that cortical morphogenesis of excystment begins with the appearance of several meridianal rows of kinetosomes in a cystic hemisphere which will become the ventral face of the future cell (Pl. II 8).

Afterwards, it can be observed a kinetosomal accumulation (Pl. II 9–10, Fig. 1) that will give place to the primordium of the AZM, which determines the anterior left area of the ventral face. In this stage, three kinetosomal dorsal rows which follow meridianal lines from the anterior pole to the posterior one and whose ends originate the caudal cirri can be observed (Pl. II 12, Fig. 1). Right and left marginal primordia are also observable.

The arrangement of the AZM kinetosomes forming membranelles begins at the anterior zone and proceeds to the posterior pole. Such arrangement of the membranelles always happened from right to left (Pl. III 13–14).

When a half of the AZM was arranged, fragmentation of the fronto-

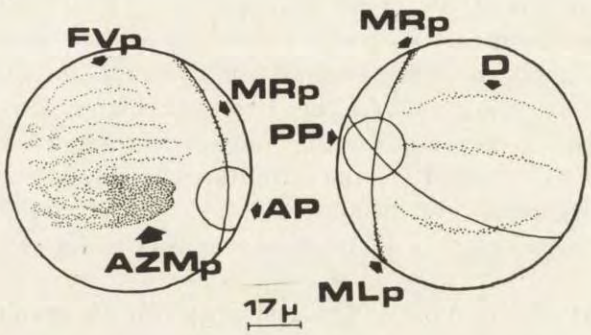

Fig. 1. Schematic figure of dorsal and ventral faces of the cyst showed in photomicrographs 10–12. AP (anterior pole), PP (posterior pole)

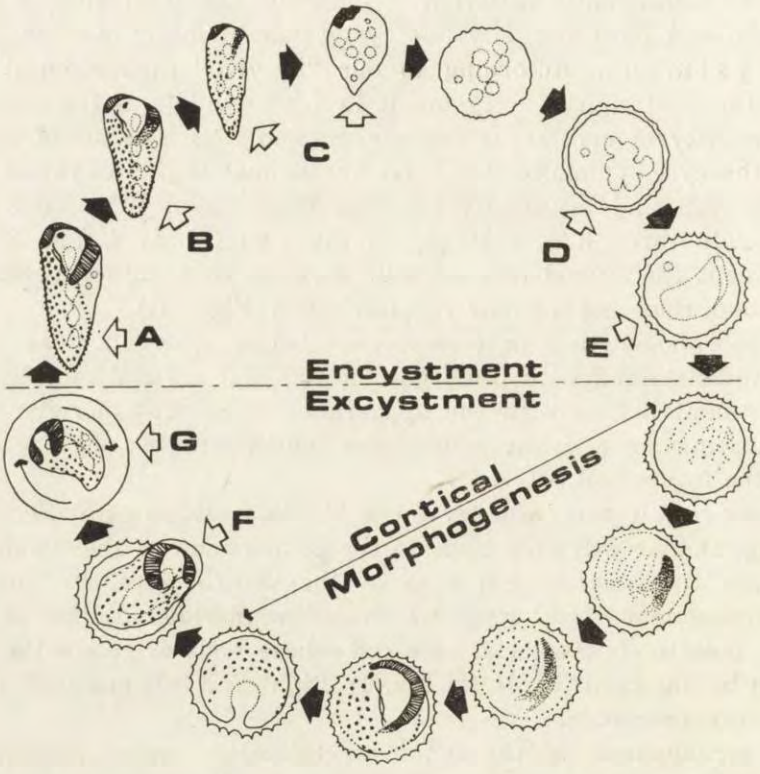

Fig. 2. Schematic figure of encystment–excystment cycle of *Laurentiella acuminata*. A — Vegetative cell. B — Resorption of ciliature. C — Macronuclear fragmentation. Micronuclear degeneration. D — Macronuclear fusion. Formation of the cyst wall. E — Resting cyst. F — Cell eclosion. G — Macronuclear amitosis. Micronuclear mitosis

ventral rows occurs giving rise to discrete basal plates (Pl. III 13). The bucal cirrus (BC) is originated by a kinetosomal proliferation at the anterior end of the endoral membrane (Pl. III 13).

After completion of the AZM, its anterior tract undergoes a curvature until it reaches the right marginal row. Dorsal ciliation is completed with the addition of several short kinetosomal rows coming from the anterior part of the right marginal row (Pl. III 15).

Simultaneously with morphogenetical processes an invagination of the dorsal face occurs, resulting in the dorsoventral flattening of the future vegetative form.

Nuclear morphological changes are observable only after emergence. The macronuclear mass divides amitotically, mostly three consecutive fragmentations occur giving four macronuclear fragments (Pl. III 16–17, Fig. 2 G), however, additional fragmentations can occur in one or more fragments resulting in cells with six, eight or ten macronuclear bodies.

Some of the micronuclei divide mitotically, methaphase being concomitant with the second macronuclear fragmentation (Pl. III 17). In 125 just excysted cells the average number of micronuclei was 4.63 ± 0.18. This value is not significantly different ($t = 0.496$, $p = 0.05$) of that found in G_1 cells (4.8 ± 0.28).

Table 2

Cytophotometrical determinations of DNA contents ($\lambda = 550$ nm) on macro- and micronuclei

Nuclear type	Stage	Statistical analyses				
		n	$\bar{X}$	σ	t	F
Macronucleus	G_1	31	155.81	18.08	1.64*	2.24*
Macronucleus	Ex	29	153.49	40.42		
Micronucleus	G_1	52	37.19	5.19	43.81**	
Micronucleus	BM	40	60.37	7.24		

Ex – Just excysted cells. BM – cells before excystment mitosis.
* Not significant at 99%
** Significant at 99%
DNA content for micronuclei are expressed as arbitrary unit, and those for macronuclei as the ratio $ma/\overline{mi}$, where $\overline{mi}$ represents the G_1 mean value of all micronuclei on the same slide.

Cytophotometrical determinations of macronuclear DNA contents in 29 new excysted cells were not significantly different from those found in 31 G_1 cells (Table 2). On the other hand, measures carried out on 40 micronuclei before excystment mitosis and on 52 G_1 micronuclei (Table 2) revealed a significative difference ($t = 43.81$) between these

populations in terms of their DNA contents, which proved that there is a micronuclear DNA synthesis previous to the mitosis occurring during excystment.

Discussion

According to Walker and Maugel (1976) the cysts of the hypotrichous ciliates can be included in two groups: NKR (non kinetosome resorbing) and KR (kinetosome resorbing). Since in the resting cyst of *Laurentiella acuminata* no infraciliature is observable upon stain with protargol, they should be included in the KR group which also includes those of *Gastrostyla steinii, Stylonychia mytilus* (Walker and Maugel 1976) and *Oxytricha fallax* (Grimes 1973).

As it is the case for other ciliates, the number of encysted cells is higher at 37°C, and the encystment is blocked at 45°C and 4°C.

Counts of the number of micronuclei in G_1 cells, resting cysts and just excysted cells suggest that some micronuclei degenerate during the encystment and that the micronuclear mean number of the vegetative cell is restored by mitoses occurring during excystment.

As reported for other protozoa (Hughes and Griffiths 1969, Giese 1973, Jehan and Dutta 1977) the encystment of *L. acuminata* is not depending on DNA synthesis but it depends on both RNA and protein synthesis (Gutierrez and Torres 1979).

During encystment of *L. acuminata* the organelles resorbed first are the UMs and then the rest of the cortical ones, the front part of the AZM being the last structure observable. These observations are in accordance with those reported for encystment of *Oxytricha fallax* (Hashimoto 1962, Grimes 1973) and for resorption of ciliature during total conjugation in *Paraurostyla weissei* (Jerka-Dziadosz and Janus 1975).

The first stages of the excystment morphogenesis observed by us in single cyst are different from those reported by Jareño (1977) and Jareño et Tuffrau (1979) for doublets. The main differences concern to localization of the paraoral membrane and to orientation of the OP with respect to the FVT primordia.

The morphogenetical pattern of excystment in *L. acuminata* is quite different from that of division (Martin 1977). As the cyst is deprived of infraciliature, obviously the oral primordium (OP) must form without association with any preexisting structure, and there is no contribution of the old cortical structures to the new ones. In contrast with the above differences concerning the origin of the primordia, there

are notable similarities between both morphogenetical processes concerning the development of such primerdia. Indeed, in both excystment and division:

(a) Bucal cirrus is originated by kinetosomal proliferation at the anterior end of the undulating membrane.

(b) The arrangement of the OP in membranelles proceed from anterior to posterior zone and from right to left, and

(c) The dorsal ciliature originates from both dorsal kinetosome rows and kinetosomes derived from the right marginal row.

As described for *Oxytricha fallax* (Grimes 1973), during excystment of *L. acuminata* both macronuclear fragmentation and micronuclear mitosis take place.

Determinations of macronuclear DNA contents in just excysted cells indicate that macronuclear DNA synthesis does not occur during the excystment, this conclusion agrees with the absence of macronuclear replication bands during such a process. However, micronuclear DNA synthesis, previous to the mitosis, has been detected by microdensitometry.

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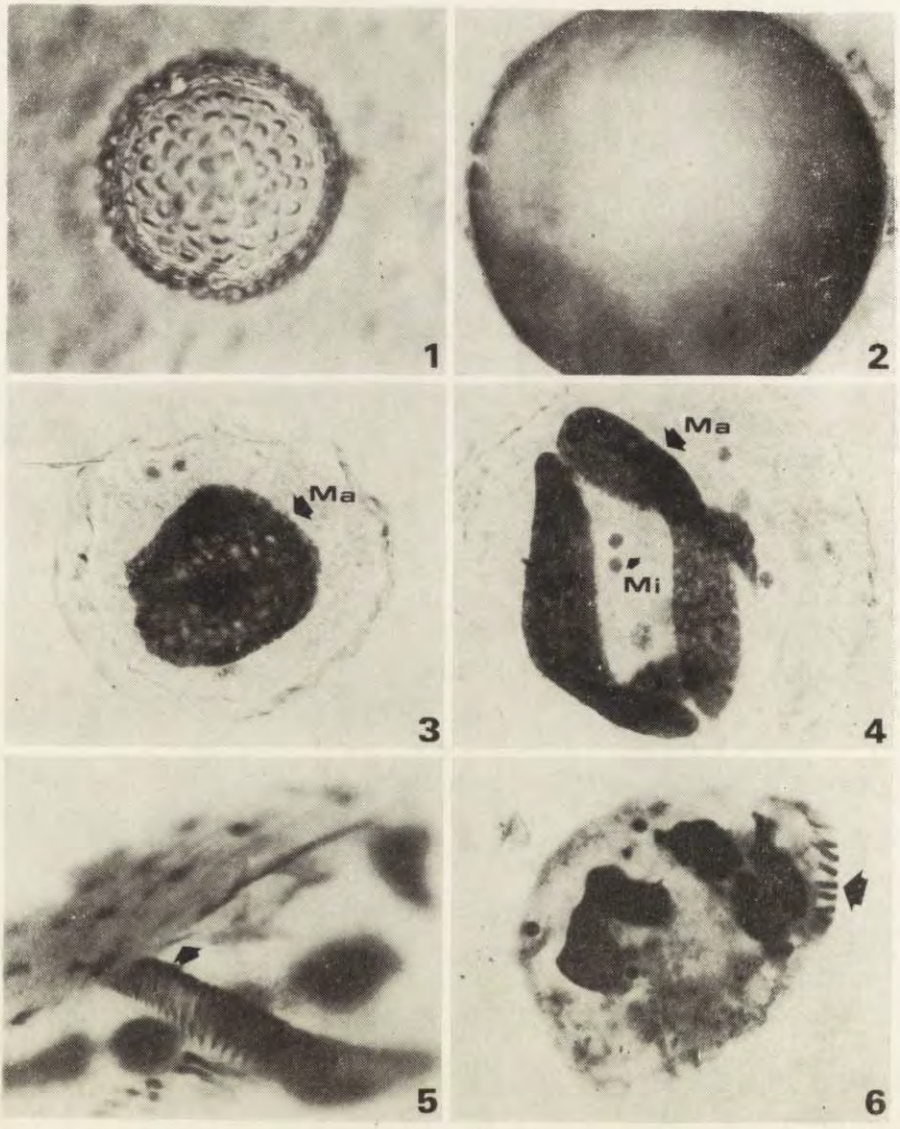
EXPLANATION OF PLATES I–III

1: Photomicrograph of a resting cyst of *Laurentiella acuminata* "in vivo". × 500
2: Resting cyst showing no ciliature nor infraciliature. Stained with Protargol. × 1000
3–4: Macronucleus and micronuclei in resting cyst. Feulgen stain. Ma — macronucleus. Mi — micronucleus, 3 — × 700, 4 — × 1000
5–6: Successive stages in the resorption of ciliature during encystment. 5 — Arrow points to the posterior tract of the endoral undulating membrane (UM). Protargol stain. × 1000. 6 — Anterior region (arrow) of adoral zone of membranelles (AZM). Protargol stain. × 800
7: Photomicrograph of the cell eclosion "in vivo". × 500
8–15: Morphogenetic processes during excystment. 8–9 — Meridianal rows of kinetosomes and kinetosomal acumulation (arrow) which from the AZMp (adoral zone of membranelles primordium). Protargol stain. 8 — × 700, 9 — × 700
10–12: Three photomicrographs showing the ventral face, equatorial zone and dorsal face of the same resting cyst. Protargol stain. 10 — Ventral face. AZMp (adoral zone of membranelles primordium), FVp (frontoventral primordia), UMp (undulating membrane primordium), MRp (marginal right primordium). × 600. 11 — Equatorial zone. MLp (marginal left primordium). × 600. 12 — Dorsal face. D. (kinetosomal dorsal rows). × 600
13–14: Arrangement (arrow) of the AZM kinetosomes. Origin of the BC (bucal cirrus). Fragmentation of the frontoventral rows (arrow). Protargol stain. 13 — × 1200, 14 — × 900. 15 — Curvature of the AZM. Note several short kinetosomal rows (arrow) that complete the dorsal ciliature. Protargol stain. FVc (frontoventral cirri), MRc (marginal right cirri). × 700
16–17: Macronuclear fragmentation and micronuclear mitosis during excystment. Feulgen stain. 16 — × 600, 17 — Micronuclear methaphase (arrow). × 900



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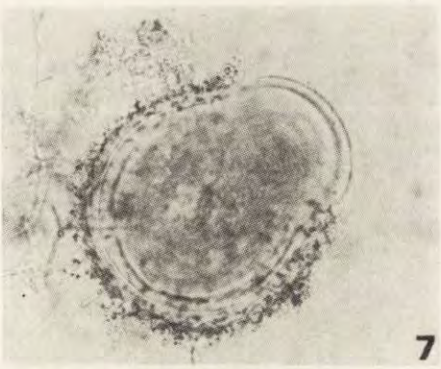

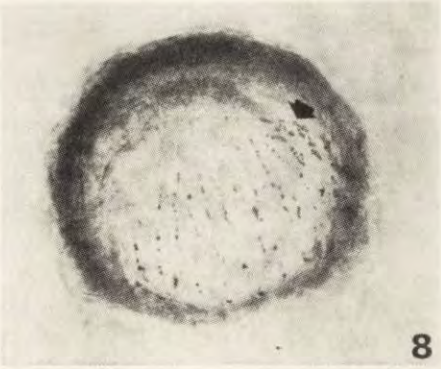

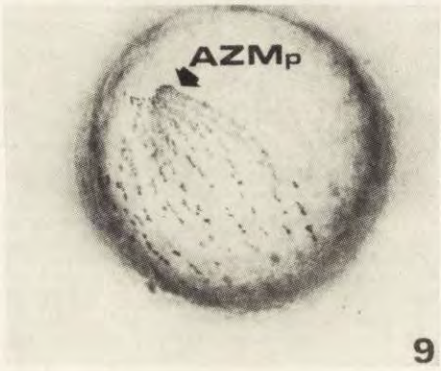

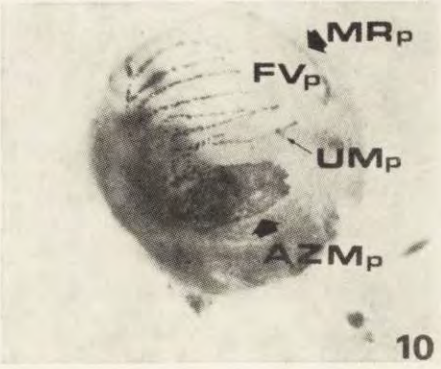

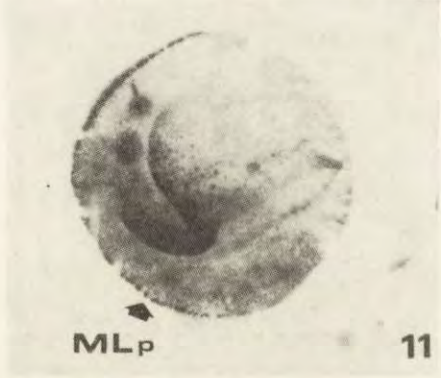

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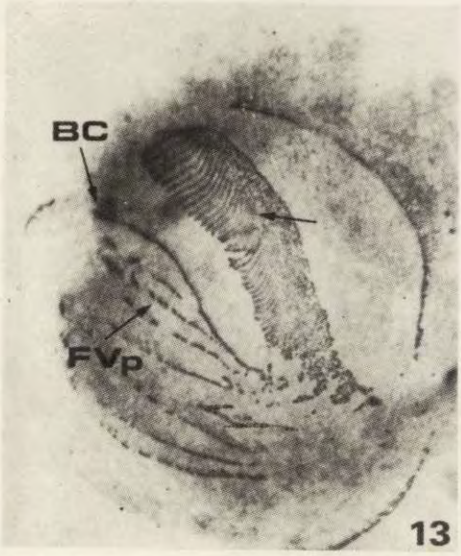

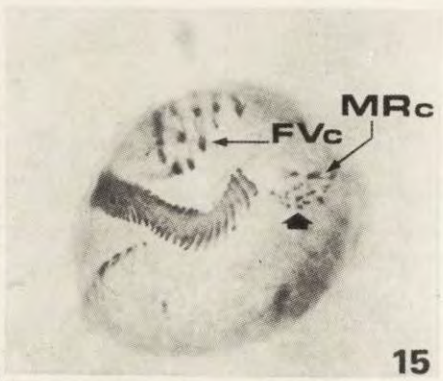

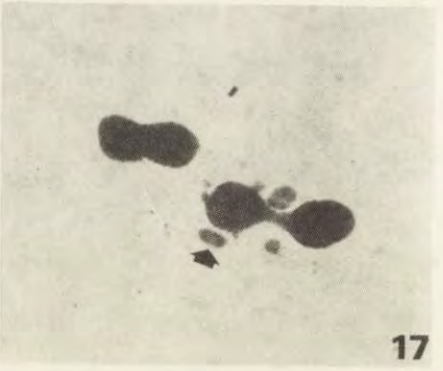

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Nuclear Phenomena of Vegetative and Sexual Reproduction in *Oxytricha bifaria* Stokes (*Ciliata, Hypotrichida*)[1]

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Synopsis. A study was conducted to describe the nuclear events occurring during the vegetative and the sexual reproduction of *Oxytricha bifaria* Stokes. The main results about the conjugative process are the following: (1) the exact timing of the different stages of the nuclear processes, (2) the demonstration that the reduction from the diploid to the haploid chromosomal set occurs during the first meiotic division, (3) the study of the fate of the four products of the synkaryon in the exconjugant.

Since the fundamental "masterpieces" of Bütschli (1876) and Maupas (1889), the nuclear phenomena taking place during the reproductive processes of ciliates have been carefully studied and precisely reported for a large number of species. The extremely complex and heterogeneous pattern of the nuclear events reported in literature in the last eighty years has been reviewed by Raikov (1972): the author reconsiders all the available data in a unitary survey, which cannot but constitute a sort of starting point for all the successive investigations.

On the other hand the problem of the exactness of the single observations previously reported cannot be disregarded, since "many peculiarities have been reported and some of these seriously conflict with basic principles of biology and require reinvestigation" (Miyake et al. 1979).

This is also the case of *Oxytricha bifaria* Stokes. The nuclear phenomena occurring during the conjugation of this hypotrich had been studied by Kay (1946) with good exactness and deep insight for

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some aspects. However, Kay reported that the reduction in number of chromosomes occurs during the second meiotic division. Such an observation contrasts with our preliminary, casual observations of the nuclear events during the conjugation of this hypotrich (Ricci et al. 1975).

For this reason, a study was planned specifically to investigate all the steps of the nuclear events occurring during both vegetative binary fission and sexual reproduction. A particular attention was paid to the exact timing of the events.

This kind of investigation was also requested by the increasing relevance of *O. bifaria* as a useful tool for research on cell interactions (Esposito et al. 1976; Ricci et al. 1980 a), induction of meiosis (Ricci et al. 1980 b) and behavior (Ricci in prep.).

Material and Methods

Clones CA-3-78 and CA-19-78 of *Oxytricha bifaria* collected in a creeck in the hills near Calci (Pisa) and belonging to complementary mating types were used.

In order to control the exactness of the timing of nuclear events, the temperature (22°C ± 1°C) used for the cultures and the experiments was constantly controlled and recorded.

To study the vegetative reproduction, the dividers were isolated as soon as they became recognizable: namely when the cells were a little larger than the normal. Groups of about 50 synchronous dividers were isolated and prepared for the cytological observations every 10 min. The cells were picked up and put onto a slide where they were fixed by Sanfelice fixative (chromic acid 1% aqueous solution: 16 parts; formalin: 8 parts; glacial acetic acid: 1 part). Afterwards the slides were processed according the classic protocol for the Feulgen staining technique. A significant improvement in the quality of the stainability of the nuclear materials was achieved by treating the cells with HCl (1 N) at 21°C for 24 h, instead of hydrolizing the DNA at 60°C for a few minutes. The slides were studied by a Leitz Orthoplan Microscope (100–1000 ×).

As to the induction of conjugation, the culturing techniques, improved according to what previously reported (Ricci et al. 1980 c), allowed us to reach an optimal standardization. This point was actually a very critical one, because it was the prerequisite for obtaining a large amount of coeval pairs, within a relatively short time interval. Pairs routinely formed within 40–60 min from the mixture of the complementary types. Groups of 80 synchronous pairs were isolated within a maximum of 5 min from the onset of cell pairing. Each group of pairs was placed into a depression and kept there until the appropriate time lag elapsed. The pairs were then processed according to the technique above reported. Slides were prepared every 30 min from 0 to 20 h from pair isolation and every 60 min from 21 to 40 h. Two rounds of experiments were run to provide an exact estimate of the timing. The times reported in the results for the different stages are the arithmetical means of the two values: it must be stressed that the difference between them never exceeded 5%.

Results

Vegetative Reproduction (Fig. 1).

A vegetative cell of *Oxytricha bifaria* is approximately 120 μm long and 40–60 μm wide. The nuclear apparatus comprehends one macronucleus divided in two parts and two micronuclei lying in a pocket on the left side of each macronuclear piece. About 10% of the specimens show a third micronucleus, usually placed between the other two. This general morphology, together with the AZM (Adoral Zone Membranelles) bordering the anterior end of the cells and continuing on the left ventral surface up to the cytostome, characterizes *O. bifaria* for about 90% of its cell cycle (Fig. 1 A). The generation time for this species has been reported to be of about 8–9 h at 22–23°C (Dini et al. 1975) and such a value proved to be rather similar to what observed during our research.

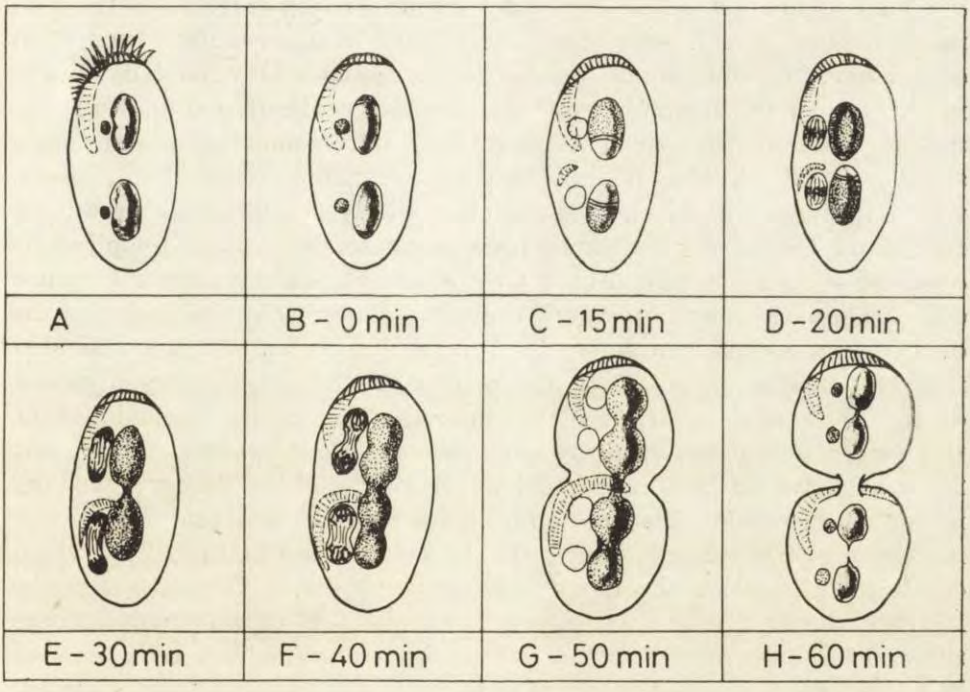


Fig. 1. A — a vegetative cell of *Oxytricha bifaria* during the interphase (dorsal view). B–H — the major stages of the nuclear events occurring during the cell division

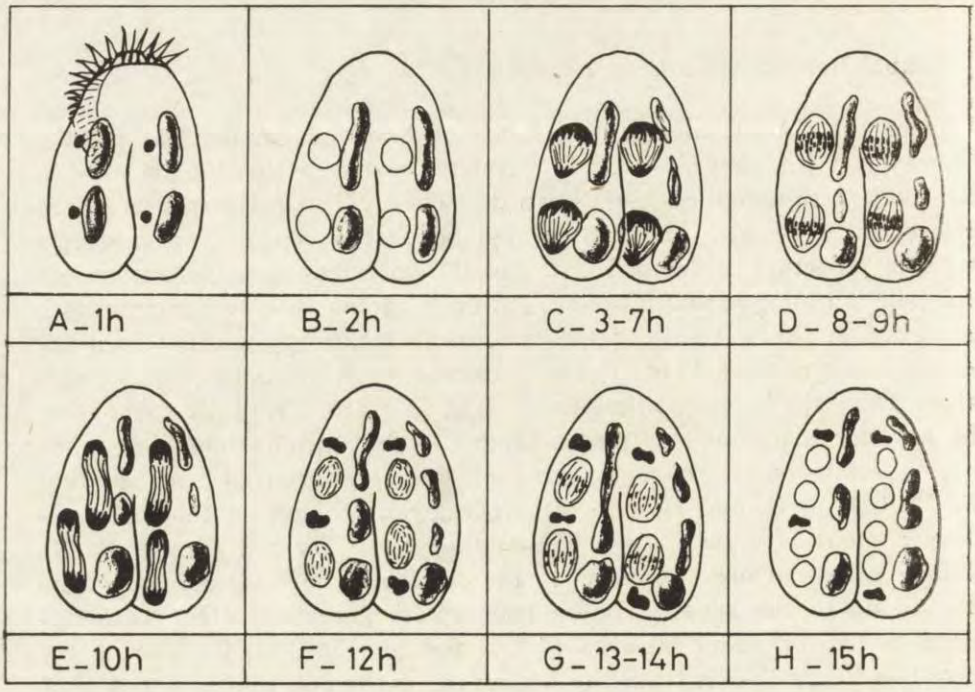
The first observable change in a cell undergoing the transverse binary fission is the appearance of two reduplication bands at the distal ends of the two macronuclear pieces (Fig. 1 B). The bands pass along the macronuclear pieces towards their proximal ends and after their passage, namely after the DNA synthesis, the macronuclear chromatin looks darker and more irregular than the normal.

When about two thirds of the macronucleus underwent the DNA synthesis (about 10–15 min after the appearance of replication bands, Fig. 1 C), the micronuclei swell, becoming very light and two times larger than the vegetative ones. At the same moment the primordium of the AZM anlage becomes visible in the left posterior half of the ventral surface. After about 20 min, the two replication bands move toward the proximal ends of the macronuclear pieces, which, in the meanwhile, became more and more close to each other. The micronuclei are now in a very typical mitotic metaphase, as one can see from the chromosomes arranged at the equatorial plate (Fig. 1 D). Because of the chromosome smallness, it was not possible to count them with a certain degree of reliability. After about 30 min (Fig. 1 E), the two macronuclear pieces, now completely dark and irregular, fuse with each other. The micronuclei are now undergoing a late anaphase. Forty minutes from the beginning of macronuclear replication (Fig. 1 F), the unique macronucleus stretches and then starts showing a main constriction that divides it into two approximately equivalent pieces, which in turn will be soon similarly divided (5'–10'), thus producing four final pieces. At the same time, a micronuclear late telophase is observable. After 50 min (Fig. 1 G) a clear cytoplasmic furrow becomes evident and the nuclear events are almost over: the macronucleus is divided now almost completely into two major pieces, which are divided into two smaller pieces. Both the primary and the secondary divisions of the macronucleus are typical amitoses. The intense stainability of the macronuclear material became less and less evident in the last 20 min: however, the macronucleus is still slightly darker than the normal macronuclei. The micronuclei are now divided completely, two in each region of the dividing cell. The primordium of the AZM, which continued elongating throughout the process (Fig. 1 C to G), strongly increases in its size and extends to the right part of what will become the anterior part of the opisthe. After about 60 min (Fig. 1 H) the two daughter cells are kept together only by a narrow cytoplasmic strand. Their morphology is characterized by the smaller value of the ratio length/width and, often, by the two macronuclear pieces not completely divided. The micronuclei, still swollen 10 min before, are completely normal, namely small and dark as they will be all the cell cycle long.

Sexual Reproduction (Fig. 2 I and II).

Once the cytoplasmic bridge is formed and one partner has rotated sideways on the other, a pair is formed: the mates lie side by side so that both can creep on the substrate. The AZM of the partners are differentially resorbed and integrated so that a single and perfectly working structure is formed. For about two hours, the general aspect of a pair is quite normal (Fig. 2 I, A): each partner has two micronuclei, as a rule on the left side of the two macronuclear pieces. Between the second and the third hour, the micronuclei swell, becoming 2–2.5 times larger than the resting micronuclei (Fig. 2 I, B). From this stage on, the anterior macronuclear pieces progressively stretch and divide, then getting scattered in the right area of the cytoplasm of each partner (Fig. 2 I, B to H, and II, I to M). No relevant change in the macronuclear content is evident until the synkaryon is formed (Fig. 2 II, N). A long micronuclear prophase of the first meiotic division lasts from the second to the seventh hour. The typical parachute stage is recognized easily for about 80–85% of this period (Fig. 2 I, C). During the next two hours (8–9 h from the beginning of the cell union; Fig. 2 I, D) the micronuclei undergo the metaphase I. Also in this case (as during the vegetative reproduction) it was impossible to count the number of chromosomes, although almost all the studied specimens showed chromatic bodies clearly and typically at the equatorial plane. We could only try to evaluate the average number (40–60) and the gross thickness of the chromosomes, in order to compare these two parameters with the correspondent two, resulting from observations of the second meiotic metaphase. A rather short anaphase ensues (Fig. 2 I, E), immediately followed by a telophase: these two stages occur in about one hour. During the next hour, of the four nuclei just formed in each partner two become typically pycnotic, namely very small and heavily stained. There is no apparent correlation between cytoplasmic position and the possible fate of the micronuclei. Whatever the distribution of the nuclei in each partner (Fig. 3) the second maturative division always occurs in not-sister nuclei.

The prophase of the second meiotic division (Fig. 2 I, F) is very short (about one hour), in dramatical contrast with the first one. It is followed by the metaphase II (Fig. 2 I, G) which is a well distinguished stage, not shorter than the metaphase I. For this reason also this stage was carefully studied in order to evaluate the number of chromosomes in *O. bifaria*. But again, the extremely small dimensions of the chromosomes allowed us only to control that both the average number of the elements at the equatorial plate (~30) and their average thick-



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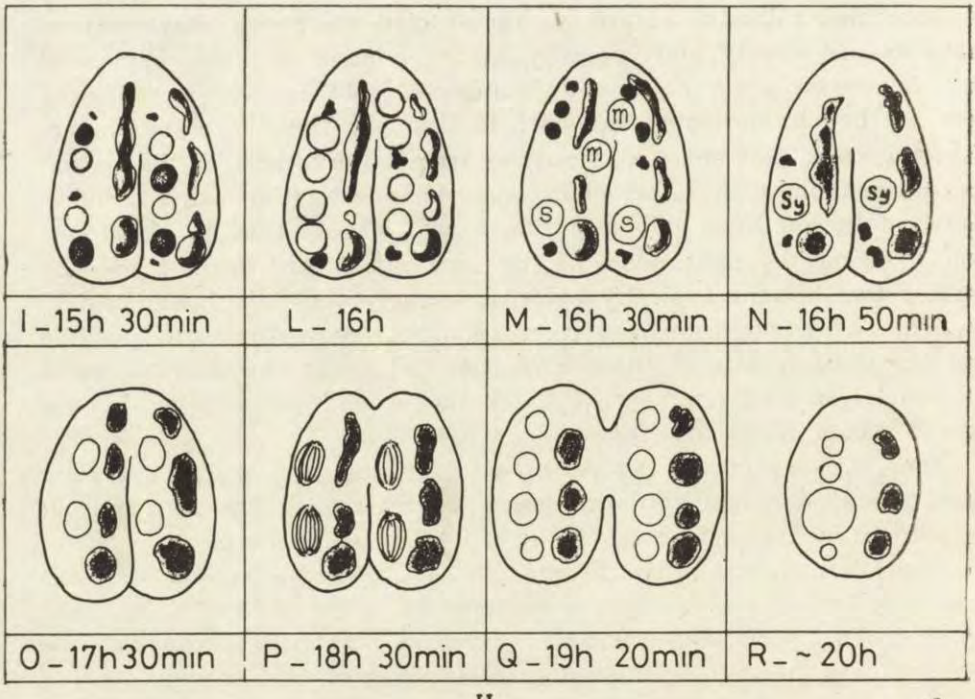

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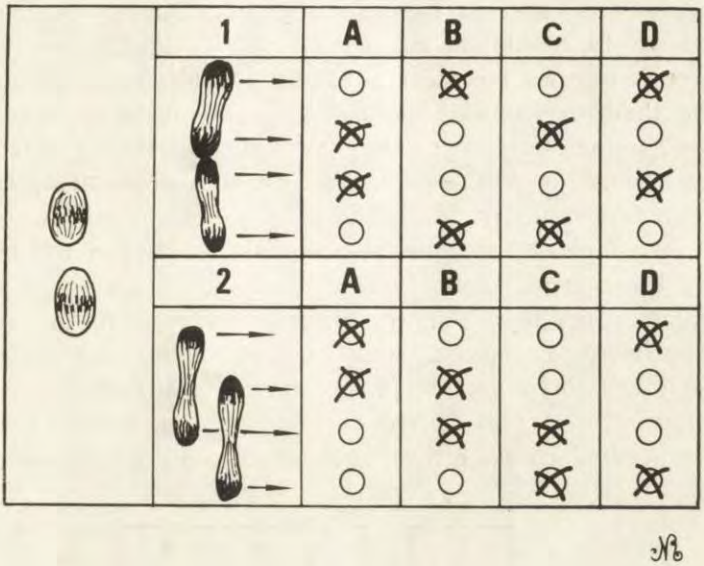

Fig. 3. The two possible spatial patterns, according to which the four nuclei, produced by a division of two micronuclei, distribute and degenerate, during the meiotic divisions. The scheme shows how always two not-sister micronuclei survive

ness are significantly smaller than during the metaphase I. After 15 h from the cell pairing (Fig. 2 I, H) four haploid nuclei are observable in each partner. Again, of the two products of the same nucleus one degenerates and the other undergoes the third division, namely a mitosis, which occurs at about 15 h and 30 min from the beginning of the process. As shown in Fig. 2 II, L, four nuclei are present again in the partners as a consequence of this third division. Of these, two sister nuclei degenerate and the other two survive, then differentiating into the migratory and stationary pronuclei. This means that the nuclear apparatuses of the two exconjugants will be genetically identical (Fig. 2 II, M).

Sixteen hours and fifty minutes from the beginning of conjugation, the synkaryon is formed and the diploid phase of the life cycle restored.

From the formation of the synkaryon on, the macronuclear pieces show a peculiar structure, with a heavily stained "chore", surrounded by a clearer area. This structure will last as long as the old macro-

Fig. 2. Schematic drawing of the major stages of the nuclear events during conjugation. I — stages from the beginning of cell pairing to the end of meiosis (A–H). II — the end of maturative divisions (I–L), the formation of synkaryon (M–N), the first four nuclei produced by its division (O–Q), the exconjugant (R). In Fig. 2 II M, "m" = migratory pronuclei, "s" = stationary pronuclei; in Fig. 2 II N, "Sy" = synkaryon

nuclear pieces will exist in the exconjugants. During the next 2.5 h (about 17 h to 19 h and 30 min, Fig. 2 II O, P, Q), two successive mitoses in each partner lead to the formation of four nuclei still larger and clearer than the normal nuclei. After about 20 h from the cell pairing, the partners separate; the exconjugants thus formed (Fig. 2 II R) have a very typical aspect: the almost completely degenerated macronuclear fragments in the right side and the four derivatives of the second synkaryon division in the central-left part of the cytoplasm. The fate of these latter nuclei was studied and it was seen that only the third nucleus, counted from the anterior end, will follow unfailingly a certain pathway, namely it will become always the macronuclear anlage. The other three may have a variable fate. However, two cases (Fig. 4, column No. 1 and 2) are overrepresented, covering about the 80% of the studied cases: either anterior nucleus degenerates and, in

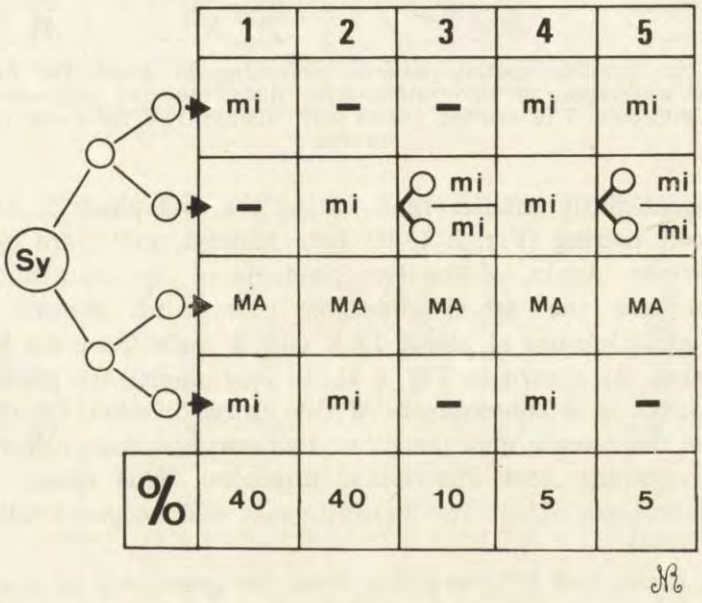

Fig. 4. The fate of the four synkaryon (Sy) derivatives is indicated: "mi" = = micronucleus, "MA" = macronucleus. In the lowest line the percentages relative to the different cases are indicated

this way, the normal aspect of the vegetative cells is restored after the first amitosis of the macronucleus. In about 10% of cases (Fig. 4, No 3), the first and fourth nuclei degenerate and the remaining nucleus undergoes a further division. In these three cases, the usual micro- and macronuclear set is restored. More rarely, however, a third micro-

nucleus is produced either by the survival of all the three products of the first two postgamic divisions (Fig. 4, No 4) or by the further division of the second nucleus, when either the first or the fourth nucleus degenerate (Fig. 4, No 5).

Whatever the story of micronuclei, the macronuclear set of the vegetative cell will be restored before the first binary fission of the exconjugant. The macronuclear anlage, rounded and transparent for about 10–15 h goes stretching and darkening for the next five hours: at the end of this period it divides in the two parts, typical of a resting cell. Thus in *O. bifaria* "clone" and "karyonide" coincide.

Summarizing all the above reported data, the conjugation of *O. bifaria* occurs in about 19 h at 22°C. This time (Fig. 5) is mainly

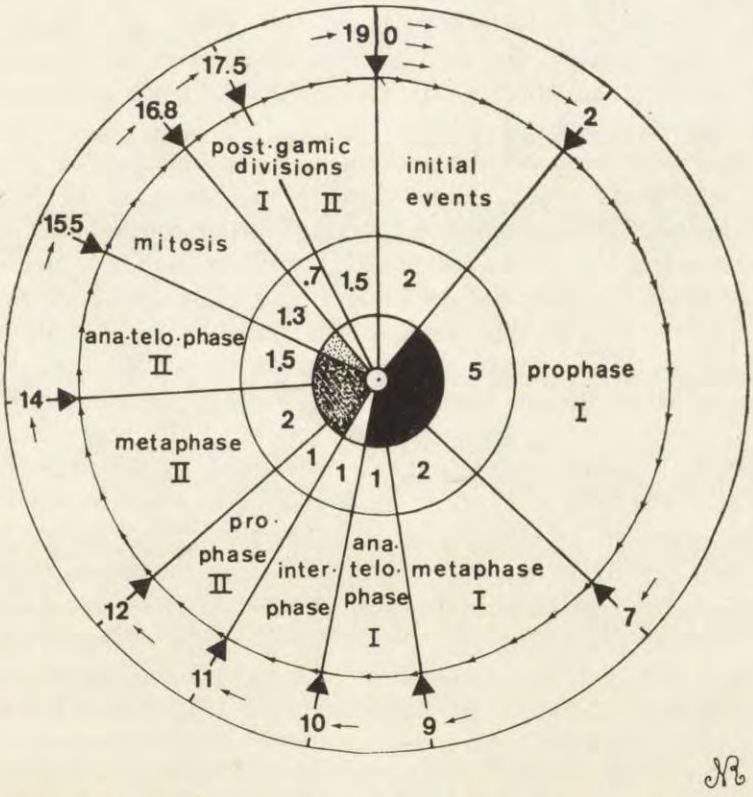

Fig. 5. Timing of the micronuclear events occurring during conjugation of *O. bifaria*. In each circular sector, from the outer to the inner part, are indicated: the progressive time (in hours); the single phase of the nuclear events, the sector refers to; the relative duration of that phase (in hours). The width of the central angle is proportional to the relative duration of each phase. The black area indicates the first meiotic division, the shadowed area refers to the second meiotic division, and the dotted area refers to the third micronuclear division

occupied by the first meiotic prophase (5 h, namely about 26%) and by the two meiotic metaphases (2+2 h about 21%) covering together about one half of all the time requested by the sexual reproduction of this species.

Discussion

The cytology of both vegetative and sexual reproduction of *O. bifaria* have been studied extensively.

As to the nuclear events occurring during the cell division we may just recall that they perfectly parallel what already known for other ciliates, namely that the macronuclear S phase slightly precedes the micronuclear mitosis. It is interesting to notice that one macronuclear replication band passes through each macronuclear piece separately. This trait proves that, although in two parts, the macronucleus constitutes a unique, physiological entity.

The research carried on about the sexual reproduction of *O. bifaria* led us to a primary result, namely the exact timing of the different stages of the nuclear phenomena occurring during conjugation (Fig. 5). The importance of this kind of data is self evident, but it is also related to the studies of the meiotic phenomena now in progress in the wake of the researches about the mechanisms possibly responsible of the induction of meiosis in this hypotrich (Ricci et al. 1980 b). The noticeable accuracy in the timing of the conjugative phenomena was allowed by the striking improvement of the culturing techniques (Siegel 1956) and of the experimental handling of the cells (Esposito et al., 1976; Ricci et al. 1980 c) in comparison with the experimental conditions of Kay (1945 a, b, 1946).

The second point which has been gained by this research is that the reduction of the diploid number of chromosomes occurs during the first meiotic division and not at the second, as Kay (1946) reported. Three major facts led us to such a conclusion: (a) the duration of the prophase I (5 h) vs the duration of the prophase II (1 h); (b) the average number of the chromatin threads and granules observable at the metaphase I (40–60) vs the average number of the same structures at the metaphase II (~30); (c) the chromatin bodies (chromatides) observable at metaphase II always slightly thinner than those (chromosomes) of metaphase I. This result can be discussed in an attempt to explain the reasons why our data apparently don't agree with Kay's point of view. Reading carefully Kay's paper and also comparing it with Gregory's results (1923) for *O. fallax* it can be concluded

that Kay's observations were correct, and the author failed only at the level of their interpretation. Gregory (1923), in fact, describes for *O. fallax* 48 granules during the prophase I and then she recognizes that they are linked two by two to form 24 chromosomes during the metaphase I; she concludes that the number of chromosomes during the anaphase I is already reduced to 12 for each pole. The same numbers are reported by Kay for *O. bifaria*, but she misinterpretes their meaning during the metaphase I, so that she can recognize the reduction only during the second meiotic division.

Moreover, today, the reduction of the number of chromosomes is known to occur during the first meiotic division and an increasing number of papers seems to prove that this is also the general case for ciliates such as *Tetrahymena pyriformis* (Ray, 1956), *Stylonychia mytilus* (Ammermann 1965), *Paramecium aurelia* (Stevenson 1972), *Blepharisma japonicum* (Miyake et al. 1979) and so on. Raikov (1972) and Heywood and Magee (1976) in their reviews clearly state that the reduction of the number of chromosomes always occurs at the first meiotic division and that this is true for Protozoa as well as for Metazoa.

One more result seems to us of particular relevance: the fate of the four products of the synkaryon. As clearly shown in Fig. 4, only the third nucleus follows a constant pathway in its development: unfailingly it gives rise to the macronuclear anlage thus suggesting a cytoplasmic control in singling out one of the synkaryon derivatives. Anyone of the other three nuclei, on the opposite, may either develop or degenerate without any particular rule: the only constant trait seems to be the capability of the second nucleus under certain, still unknown conditions, of undergoing a third division.

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Autofluorescence of Axenically Cultivated *Paramecium aurelia*

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Synopsis. Cells of stock 299 s *Paramecium aurelia* cultivated axenically exhibit fluorescence in region of 405–600 nm with maximum at 440–450 nm. This autofluorescence is diminished significantly following starvation in Tris buffer.

Spectrofluorometric analysis of spent and fresh axenic medium revealed fluorescent compounds with emission peaks at 440 nm and 507 nm thus indicating that the uptake of nutrient medium is a source of cellular fluorescence. The appearance of fluorescence peak at 440 nm in spent Tris buffer in which the cells have been maintained suggests that these fluorescent substances are subsequently removed from the cells during starvation.

Autofluorescence has been demonstrated in plant tissues and in a variety of animal cells (Udenfriend 1962, Pearse 1972, Prenna et al. 1974, Prenna et al. 1977, Poccia et al. 1979, Aubin 1979, Benson et al. 1979) but it has not been reported in *Protozoa*. Detailed studies on autofluorescence of mammalian cells recently presented by Aubin (1979) and Benson et al. (1979) demonstrate that emission in spectral region of about 445 nm and 500–600 nm is probably due to intracellular nicotinamide adenine dinucleotide (Aubin 1979) and flavoproteins (Benson et al. 1979, Aubin 1979) respectively.

During our studies on supravital labelling of *Paramecium* surface proteins with cycloheptaamylose—dansyl chloride complex (Wyroba et al. in preparation) we have observed that the ciliates freshly taken from the axenic medium displayed their own fluorescence in the region of 400–600 nm. Its intensity decreased significantly in star-

ved cells suggesting that autofluorescence may be due to the uptake of medium. Thus to characterize this phenomenon the emission spectra of cells, axenic culture medium, spent medium and starvation buffer solutions have been performed using high-sensitivity fluorometric equipment.

Material and Methods

Stock 299 s of *Paramecium aurelia* has been cultivated in standard axenic medium according to Soldo et al. (1966) in darkness at 18°C. Cells were collected by centrifugation at 800 g for 1 min, washed with migration salt solution — MSS (Soldo et al. 1966) and twice with 0.005 M Tris-HCL buffer containing 0.001 M $CaCl_2$, pH 7.6. The density of ciliates was about $1.5–2.5 \times 10^4$ cells per ml. The fast filtration of cell suspension on 15 μm nylon sieve — applied by us in CDC method (Wyroba et al. – in preparation) — has been used to remove any fluorescent contamination derived from culture medium. Following filtration cells were living and moving. Some cells have been examined immediately after first washing whereas the others have been maintained in Tris buffer for the defined time intervals before measurments of fluorescence. The filtered cells were analyzed fluorometrically on microscopic glasses as dried samples.

The emission spectra were performed by means of the Leitz microspectrograph equipped with a single photon counting system (Cova et al. 1974) and operating in a digital lock — in mode with automated and repetitive scanning of the emission wavelengths (Giordano P. et al. – unpublished). A Xenon lamp Osram XBO 75W was used as the light source. The emission spectra ranged from 405 nm to 700 nm and were obtained with excitation wavelength at 370 nm (slit width of 70), dichromatic mirror TK 400 and Leitz objective 40 X.

The axenic medium, spent medium and spent Tris buffer (in which the cells have been maintained) were filtered three times through Whatman filter paper before spectral analysis. Emission spectra of the above mentioned solutions were measured by an Applied Photophysics spectrofluorometer in the range of 390 nm–646 nm with excitation wavelength at 366 nm.

All measurements were made at room temperature.

Results

The cells freshly taken from the axenic medium (6-days old culture) and washed once with MSS exhibit rather a uniform fluorescence (Pl. I 1) with some more bright structures. Among them the food vacuoles displaying yellowish fluorescence can be recognized when visible light was used in microscopic observations. Spectral analysis of these cells at the excitation wavelength 370 nm revealed an emission spec-

trum with a single fluorescence peak located in the 440–450 nm region (Pl. II 2 a). This autofluorescence can be quenched by KJ: when drops of its solution have been added the fluorescence intensity decreased rapidly and the cells appeared almost completly fade in fluorescence microscope.

When the ciliates after normal washing procedure have been maintained in 0.005 M Tris-HCl buffer containing 0.001 M $CaCl_2$ (pH 7.6) their autofluorescence was much smaller depending on the duration of starvation. Plate II 2 b demonstrates the emission spectrum of cell starved in Tris buffer for 5 h: the shape of spectrum is similar to that in Pl. II 2 a but the intensity of fluorescence is lower. Autofluorescence of cells maintained in buffer for 24 h is hardly visible in fluorescence microscope (not shown) but it may be clearly revealed in microspectrograph (Pl. II 2 c): emission spectrum in this case is flat and the intensity of fluorescence is very small.

The observed higher intensity of autofluorescence of cells freshly collected from the culture than that of starved ones suggested that this emission may be due to the medium uptake. In order to explain the appearance of autofluorescence the examinations of culture media, spent culture media, buffer solution and spent buffer solution have been performed in wide spectral region from 390 nm to 646 nm. The emission spectra of sterile axenic media are presented in Pl. II 3 a, b. Two fluorescence peaks can be observed: the first at 440 nm and the second at 507 nm. The difference in the intensity may be due to the conditions of sterilization: the medium analyzed in Pl. II 3 a has been autoclaved in 750 ml erlenmyer flask closed with cotton-wool stopper while that presented in Pl. II 2 b in 5 ml tube firmly capped. Emission spectrum of spent culture medium is shown in Pl. II 3 c: the shape of the curve and the location of the peaks corresponds to those obtained for fresh medium. (For measurements of media correction has been done for bidistilled water).

Spectral analysis of spent Tris buffer in which the ciliates have been maintained for 48 h revealed one major fluorescence peak at 440 nm whereas the second maximum almost completely disappeared (Pl. II 3 d). Fresh Tris buffer has not been fluorescent. The cells starved for 48 h in Tris buffer exibit a faint fluorescence with the emission spectrum similar to that demonstrated in Pl. II 2 c. It should be noted that the shape of emission curve of cells freshly taken from the medium (Pl. II 2 a) is very similar to that of spent Tris buffer (Pl. II 3 d). This indicates that the substances fluorescent in the cell, uptaken from the nutrient medium, are subsequently removed to the washing and starvation solution.

Discussion

The presented results indicate that axenically cultivated *Paramecium* cells exhibit the autofluorescence in the spectral region of 405–600 nm with the maximum in the range of 440–450 nm. This autofluorescence is diminished markedly following starvation in Tris buffer: the intensity of cell fluorescence after 24 h of maintenance in buffer is 3-fold less than that of ciliate taken from the nutrient medium. The analysis of axenic medium revealed fluorescent compounds with 2 maxima of fluorescence: at 440 nm and 507 nm. Thus the uptake of fluorescent substances present in the medium seems to be a source of cellular autofluorescence. These fluorescent compounds are most probably vitamins added, mainly riboflavin in free or bound (with proteoses or peptones supplied also in medium) form as can be concluded from the spectral analysis (Udenfriend 1962). Riboflavin amounts to 11% of all the vitamins added to axenic medium. Fluorescence peak of riboflavin at 440 nm when excited at 360 nm has been recently reported by Aubin (1979). Autofluorescence of mammalian cells observed in the same spectral region has been reported to arise from the intracellular nicotinamide adenine dinucleotide (NADH), flavin coenzymes and flavoproteins bound in the mitochondria (Aubin 1979). In *Paramecium* the nature of autofluorescence seems to be exogenous. The fluorescence in mammalian cells was found to originate in discrete cytoplasmic vesicle-like regions whereas in *Paramecium* it seems to be rather homogenous all over the cell.

It should be noted that decrease in fluorescence intensity observed in starved cells corresponds to appearance of fluorescence peak at the same wavelength in buffer solution used as starvation medium. This correlation indicates that fluorescent compounds uptaken (in excess?) by the ciliates from the nutrient medium have been subsequently removed from the cells during starvation.

Concluding it may be stated that in experiments employing fluorometric techniques on axenic protozoan cultures the influence of cellular fluorescence should be carefully considered.

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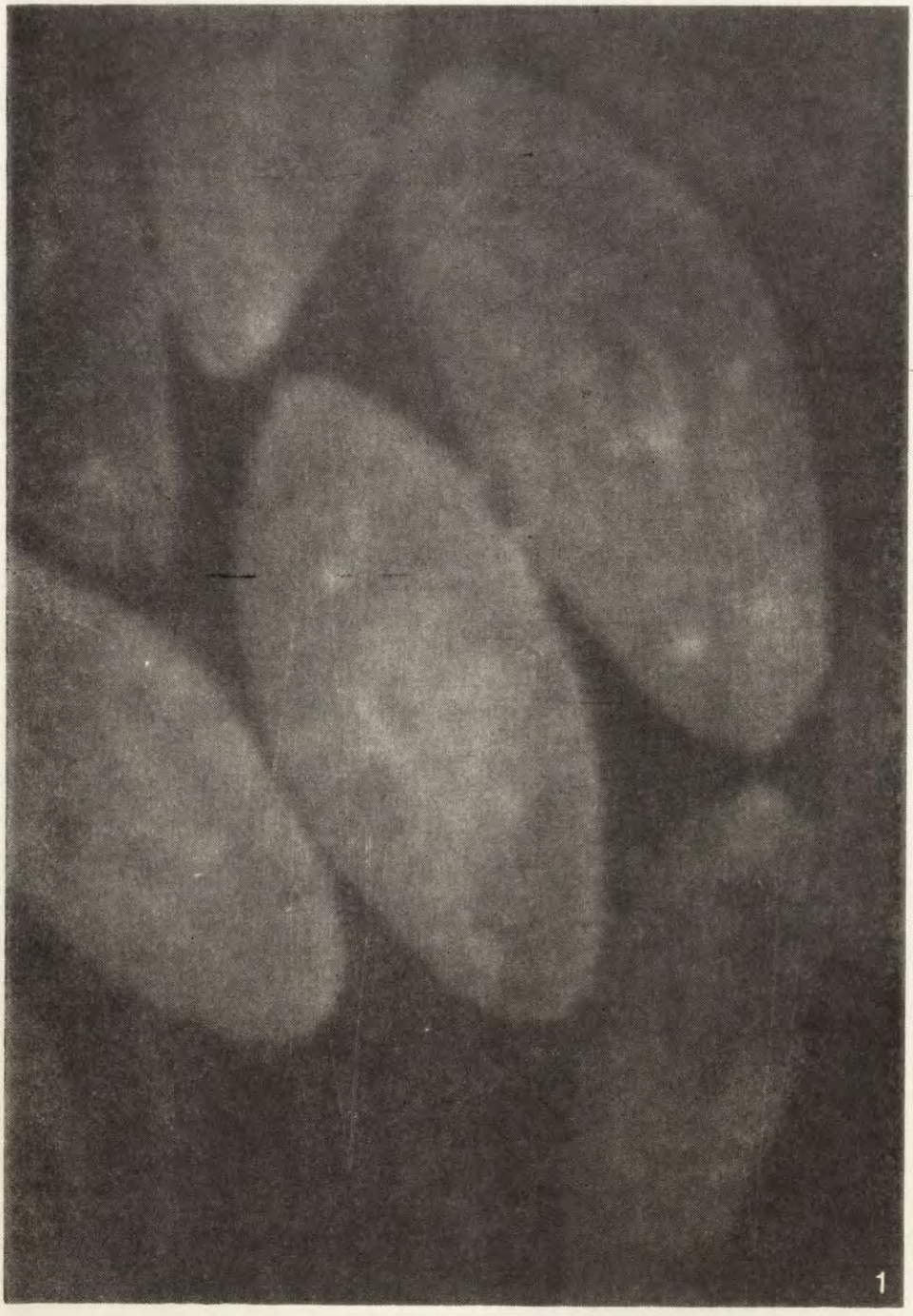
EXPLANATION OF PLATES I–II

1: Autofluorescence of *Paramecium* cells freshly taken from the medium and washed with MSS visualized in Leitz fluorescence microscope. (Cells viewed with excitation filter BG 12, dichroic mirror 3, TK 495 and emission filter K 510 nm). Exposure time 120 s. – 1000 ×

2: Autofluorescence of *Paramecium* cells–emisision spectra of ciliates freshly taken from culture medium and starved ones, a — cells freshly taken from the medium; b — cells starved for 5 h in Tris-HCl buffer; c — cells starved for 24 h in Tris-HCl buffer. F. I. — fluorescence intensity (expressed in arbitary units). Photograph of the oscillographic display of microspectrofluorometer is shown. Six scans of each measurement are presented

3: Fluorescence of nutrient media and starvation buffer — emission spectra, a, b — axenic medium (see also Results) c — spent axenic medium d — spent Tris-HCl buffer in which cells have been maintained for 24 h. Photograph of the oscillographic display of apparatus is presented



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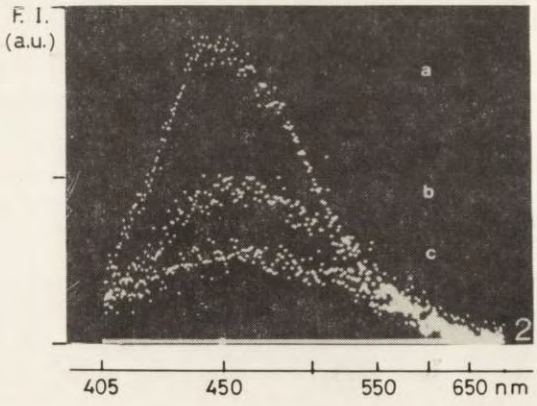

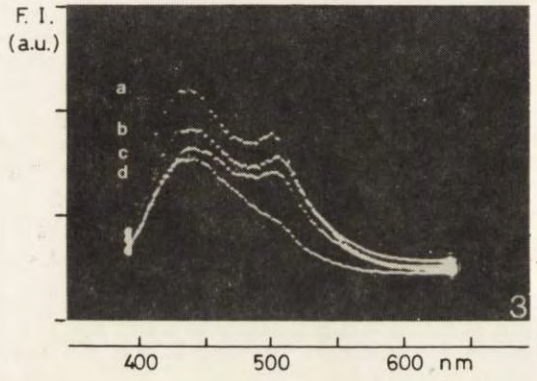

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Electrical Properties of Cell Membrane in *Stentor coeruleus*. III. Influence of Changes in Temperature on the Membrane Permeability to Ions

Received on 22 November 1980

Synopsis. The effects of varied temperature on membrane input resistance (R_m) was measured in the ciliate *Stentor coeruleus*. The general effect of warming of culture medium was to depolarize the cel membrane and decrease the membrane resistance often to one-half of the value recorded at lower temperature. Assuming that membrane potential is determined mainly by K+ and Na+ ions, calculations of the membrane permeability to particular ion were made from the data on change of R_m with temperature alteration from 8 to 18°C. The obtained results showed that depolarizing effect of temperature increase arised from a higher temperature coefficient of sodium permeability compared with those of other ion species.

Recently it has been shown that the electrical potential difference across the cell membrane of *Stentor* changed with rising ambient temperature (Fabczak 1980 b). The generally accepted mathematical description of membrane potential difference generation in muscle or nerve cells presumes the existance of an electrochemical gradient through the membrane and that the permeability has an appropriate value for particular ions (Goldman 1943, Hodgkin and Katz 1949). Thus, in view of the foregoing, the value of the potential difference on the semipermeable membrane should be a linear function of the absolute temperature. This, however, is not completely confirmed in the case of potential-temperature relation for the protozoan cell membrane. Therefore the suggestion advanced by Hodgkin and Katz (1949) that the temperature dependence of membrane potential

may be due to changes in the individual ion permeability with temperature alteration can not be ruled out.

The experimental data presented in this paper concern mainly temperature dependence of ionic membrane permeability in the cells of *Stentor*.

Materials and Methods

The procedures of growing and preliminary preparation of *Stentor coeruleus* to experiments as well as electrophysiological measurement method have been described in detail in earlier papers (Fabczak 1980 a,b).

Additionally, in the present study the liquid junction potential change with temperature for various tested external media was investigated. The noticeable changes of this potential were not observed within the whole range of temperature applied. These observations are in a good agreement with similar data reported by other authors (Dalton and Hendrix 1962, Senft 1967).

The determination of the membrane resistance, R_m, (and conductance, g_m) were made with square pulse analysis and an ascending current ramp was utilized (Fatt and Katz 1951, Starzak et al. 1977). The recordings of slow electric transients delivered by voltage and current microelectrodes were carried out by the use of a high input impedance electrometer (10^{14} ohms) and a chart recorder.

Results

In order to check whether the cell membrane depolarization on warming is a result of the conductance changes, each cell of *Stentor* was subjected to a temperature rise from 8 to 18°C and measurements of R_m were made at these two temperature settings.

As it is shown in Fig. 1, with temperature increase of 10°C, for all cells in a good physiological condition, R_m, was reduced to one-half of the value of the initial recordings, and resting membrane potential showed at the same time a 30–35 mV depolarization (Fabczak 1980 a). After temperature shifting, both R_m and membrane potential maintained their new values as long as appropriate temperature was held.

Also the ratio between R_m (or g_m), calculated as the slope of a current–voltage relationship, obtained at 8 and 18°C significantly changed when the culture solution was substituted by external medium with altered ionic composition. Figure 2 presents the results of the measurements of relative membrane conductance, g_m, in terms of the slope of voltage–current relations, for three protozoan cells in three different tested solutions: I (3 mM KCl), II (5 mM NaCl) and III (no

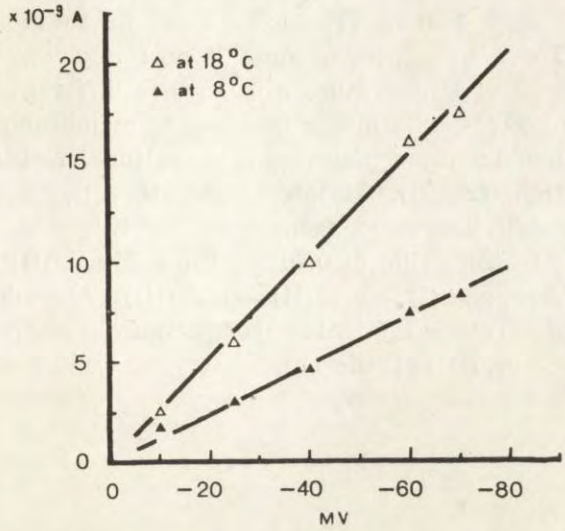

Fig. 1. Steady-state voltage–current relations obtained in *Stentor coeruleus* with culture medium for one cell at 8 and 18°C. The slopes of curves generated by hyperpolarizing current ramp, represent membrane conductance. The duration of the ramp stimulation was 3 s

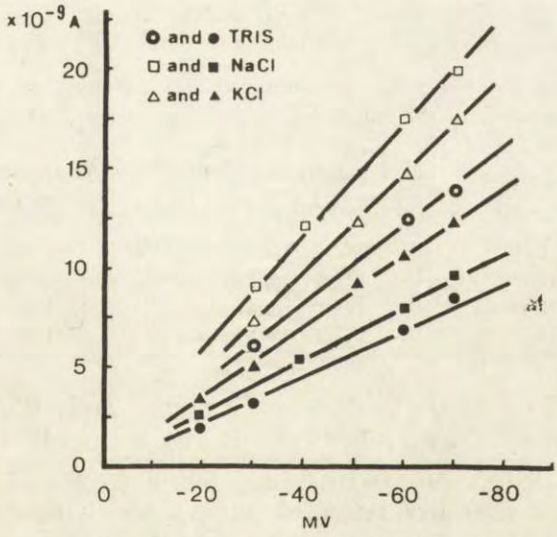

Fig. 2. Steady-state V-I plots obtained in *Stentor coeruleus* with three different solutions at 8°C (filled points) and 18°C (opened points). Data are collected from three cells. Membrane potential difference was kept at the same level by continuous current injection, to balance the resting potential changes as the result of external solution replacement. The curves were produced in the same manner as those in Fig. 1

KCl, no NaCl; only 1 mM Tris-HCl). Each of these solutions were osmotically balanced by a proper amount of 1 mM Tris-HCl — buffer of pH 7.3. The rise of temperature alters g_m in different degree for the particular ionic species. The major increase in membrane g_m was found with temperature during replacement of culture medium by sodium containing solution (i.e., II). At low temperature the slope of voltage–current relationship decreased from 0.68 for K^+, 0.51 for Na^+ with further decrease to the value of 0.46 for the solution III; i.e., membrane conductances were g_m (I) > g_m (II) > g_m (III). Accordingly, at 18°C the order of relative ionic conductance decrease becomes g_m (II) > g_m (I) > g_m (III) (Table 1).

Discussion

According to temperature factor occurring in Goldman's equation (Goldman 1943) the value of electrical potential difference across the cell membrane should undergo changes amounting to 3% with a temperature shift of 10°C, under the assumption, of course, that the permeability itself does not change. Changes in the electrical membrane potential of this order have been observed for several cell preparations

Table 1

Effect of various external solutions on the relative individual conductances of the cell membrane obtained at 8 and 18°C

Solution	Conductances (slopes) g_m(18°C)/g_m(8°C)	Temperature coefficient
I (K^+)	0.97/0.68	1.42
II (Na^+)	1.11/0.51	2.17
III (Tris)	0.91/0.41	1.98
culture	1.02/0.57	1.80

(Ling and Woodbury 1949, Nastuk and Hodgkin 1950, Jenerick and Gerard 1953, Kerkut and Ridge 1961). Such electrical behaviour, however, is not a general rule in the cell membranes since in other reported studies the temperature nonlinearity was noted in temperature — potential relationship (e.g., Hodgkin and Katz 1949, Carpenter 1967). The result of experiments described here demonstrates that the magnitude of temperature dependence of resting membrane potential of *Stentor* can not be explained on the basis of proportionality to the absolute temperature as well.

It is known from the abundant research literature concerning similar relations in muscle and nerve cells that the divergences from mathematical formula occurring in such phenomena can be interpreted in two ways: either these temperature dependence of membrane potential arise owing to the direct modification of the permeability to the particular ions with temperature, or by activation of the cellular metabolic processes taking part in the electrical phenomena by temperature (Carpenter and Alving 1968, Baker et al. 1969, Senft 1967, Ayrepetyan 1968).

In the present study the possibility that metabolically linked processes may have same effect on the value of membrane potential difference was not investigated. However, reported here observations compiled in Table 1 illustrate clearly that a rise of temperature in the cell environment greatly enhanced the ionic conductance for all three solutions but in different degrees for particular ion species (Fig. 2). The greatest positive correlation found between the membrane conductance increase to sodium ions and the membrane potential with warming allow to advance the suggestion that, under the experimental conditions applied, the depolarization of the membrane potential depolarization at the rest resulting from the temperature rise occurs among other things as the consequence of unequal changes in permability of the cell membrane of *Stentor* to the particular ions. So, the observations that membrane conductances measured as a function of temperature upon the voltagecurrent relationship of the cell membrane, should be taken into account at the description of the process governing the ionic mechanisms on the protozoan cell membrane.

This experimental finding is in agreement with the similar data obtained for the giant axon of squid (Hodkin and Katz 1949) and *Aplysia* giant nerve cell as well (Marchiafava 1970) — where it was stated that the temperature coefficient for the sodium transfer across the nerve membrane at resting state is higher than for potassium.

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Toxic Action of Colistin and Penicillins V and G on *Tetrahymena*. I. Lethal Effect and Influence on Multiplication[2]

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Synopsis. Toxicity of colistin was about 100 times higher than that of penicillins. It was proved that penicillin V was detectably more toxic than penicillin G. On the basis of a comparison of antibiotic toxicities against non-initiated and initiated cells and because of differences in their antimultiplicatory effects, different mechanisms of action for colistin and penicillins in tetrahymena as an animal-model cell are postulated.

The purpose of our investigations was a comparison of some biological effects of the selected β-lactamic and polypeptidic antibiotics, as well as an attempt to determine the aplicability of tetrahymena in screening the action of drugs of similar composition but including various chemical groups (penicillin G and penicillin V).

All the three antibiotics used in the experiments are peptidic-type compounds. As it is known, penicillins specifically block the 3rd stage of the bacterial cell wall biosynthesis. By "specific action" we understand a directed damaging action of the drug against physiological processes or structures occurring in the biological object under investigation. Since no bacterial cellular wall-type structures have been found in tetrahymena, the observed effects of treatment with penicillins will be considered as a sign of their non-specific action. Penicillins are considered by pharmacologists as ones of the least toxic antibio-

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tics for animals, and that is why they are so broadly applied in therapy and as an antibacterial agent in different animal and protozoan cell cultures. The difference in chemical constitution between penicillin G and penicillin V has no effect on their antibacterial specific activity or on the character of the so-called "side-effects" in therapy. It is claimed that this difference causes only a higher stability of penicillin V in acidic environment than that of penicillin G. However, in range of pH 6–7, both penicillins are showing similar stability in solutions.

Colistin, an antibiotic in the polypeptide group, has been provisionally considered as an agent acting specifically because of its detergent-type action against cell membranes. It is generally suspected that the polypeptide antibiotics are disturbing bacterial building-up or recomposition of the used-up parts and components of cytoplasmic membranes. Polypeptidic antibiotics are much more toxic to animals than penicillins. Penicillins are bound stronger by blood proteins than colistin, however, both types of antibiotics are weakly bound in pepton (Korzybski et al. 1977 and Kuryłowicz 1979). No reports on the action of colistin against tetrahymena have been found. Gross (1955), Nemeth and Csik (1961), and Nickerson and Rij (1949) claimed out the influence of penicillin on cellular metabolism of *Tetrahymena pyriformis*, however, at the present stage of experimental protozoology, these works seems to raise today methodical objections.

Materials and Methods

Investigations were carried out on *Tetrahymena pyriformis* GL-C, the strain was obtained from Nencki Institute. The cells were cultivated axenically in 1.5% proteoso-pepton (Difco) with 0.1% of yeast extract (Difco) and supplemented with salts according to Plesner et al. (1964) at 28°C. Stock cultures ("A" line) were grown in test tubes containing 5 ml of the medium and transferred every second day. One day before each experiment, the cells were transferred from a 2 days-stock into 200 ml Erlenmayer flask containing up to 25 ml of the medium ("B" line). They were incubated overnight without any agitation and then used for experimental inoculations. The depth of those culture layers were less than 0.6 cm. It was previously examined that, under the described conditions, agitation had no acceleratin effect on the dynamics of growth. The total density of cultures before and during the experiments were tested by electronic counter which was built according to the data made available to us by dr L. Rasmussen (personal communication). Antibiotics (pure substances): colistin, penicillin V and penicillin G were obtained from the producer, "Polfa" Pharmaceutic Works. Colistin was used as a sulphate salt, molecular weight 1400 and specific antibacterial activity 18.309 units/mg. For a better comparison, both penicillins were used as potassium salts. The difference in chemical constitution of penicillins confines

to one oxygen atom (the difference between benzyl- and phenoxymethyl-groups). Molecular weights and antibacterial activity for penicillin V are 388.51 and 1520 u/mg, and for penicyllin G — 372.51 and 1614 u/mg, respectively. Stock antibiotic solutions were prepared directly before each experiment.

Lethal Effect

The cells used for experiments were taken from the stationary phase of cultures. The antibiotics were tested on the cells suspended in the medium and in each of the two starving solutions (1mM Tris-HCl+1mM $CaCl_2$ pH 7.3 and standard Dryl's solution) (D r y l 1959).

The cells were separated from the medium by centrifuging (3 times) and then incubated in starving solutions for 10 h at 28°C. Whenever the test was carried out in the basal medium, the exhausted medium obtained from the culture of the same physiological condition as that under examination was used for making the antibiotic solutions. Serial antibiotic dilutions were mixed with the cells suspension in the ratio 1 : 1. The test was carried out in depression slides in the total volume of each sample equal 1.0 ml. The incubation time was arbitrarily fixed as 20 h at 28°C. The degree of survival was estimated and the values of lethal dose — 50% (LD-50) were based on lethal curves obtained for three repetitions of the experiment.

Effect on Cell Multiplication

The experiments were carried out on mass cultures in the logarithmic phase. The experimental Erlenmayer's 200 ml flasks including 23 ml of the medium were inoculated with 1 ml of "B" line cells. The densities obtained after inoculations stayed within the ranges 1.0×10^4 to 1.5×10^4 cells/ml. The experiments were carried out in water bath (28°C) with aeration by agitation. Under the described conditions, the lag-phase and the growth rate acceleration period terminate in a time shorter than 3 h. The antibiotics diluted in distilled water were added to the culture in 1 ml volume, 3 h after inoculation. For control, 1 ml of clean, distilled water was added though it was ascertained that this had no effect on the multiplication rate of the cells if the added water had the same temperature as the culture. 1 ml samples were counted on an electronic counter: in "O" point (directly after administration of the antibiotics), and then after 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 h. In all the experiments, the number of cells in the end point of experiment (5.0 h after administration) was always lower than 1.0×10^5 cells/ml, as this density is always considered as the upper boundary for the logarithmic phase.

Results

The results of the experiments on the antibiotics toxicity are shown in Table 1. In the given test conditions, the division index (highest in PPYS) was detectable but essentially lower than 0.01%. The comparison of LD-50 for the antibiotics acting on non-starved cells showed that colistin was more toxic than both penicillins, although

Table 1

Toxicity of colistin and penicillins for *Tetrahymena pyriformis*

	Media		
	PPYS	TRIS	DS
colistin	6	0.11	0.08
penicyllin V	16	23	30
penicyllin G	24	26	32

Toxicity was measured as a lethal doses — 50% (LD-50), during 20 h of experiment in different media: PPYS — basal growing medium, TRIS — 1 mM Tris-HCl+1 mM $CaCl_2$ pH — 7.3, DS — standard Dryl's solution. LD-50 concentrations are expressed in mM. See the text for further explantation.

the differences were slight (2 and 4 times, respectively). It is, however, worth to remember that all the three antibiotics proved about 1000 times less toxic for *Tetrahymena pyriformis* than for the sensitive bacterial strains. A comparison of non-starved and starved cells sensitivities (in both starving solutions) to the same antibiotic showed a valid difference. Colistin proved about 50 times more toxic for starved cells. However, to penicillins the starved cells seemed to be even more resistant than the non-starved cells. Moreover, it seems that irrespective of the test conditions, penicillin V was more toxic than penicillin G. Sharpest curves of lethality were obtained only for penicillins and only when they were tested in Dryl's solution. The results the experiments on the antibiotic action against multiplication of exponentially growing cultures of *Tetrahymena pyriformis* are shown in Fig. 1. It was simultaneously examined that the addition of antibiotics in the tested range of concentrations to the cultures, lowered pH of the medium less than 0.1 of pH even in the highest concentrations tested.

It is possible to isolate and analyze independently few different effects of a chemical agent on the investigated process: difference between ranges of active concentrations, the presence of lag phase and its duration as function of concentration and, at last, possibility of complete recovery of the doubling rate after the recovery of dividing. All the examined antibiotics in sublethal concentrations exerted an influence on cell multiplication.

With the identical system of antibiotic dilutions, colistin showed highest and the penicillin G lowest range of active concentrations. All the antibiotics caused the appearance of lag phase.

The duration of lag phase was 2–3 times longer for colistin than for both penicillins. For colistin poor dependence of the lag phase

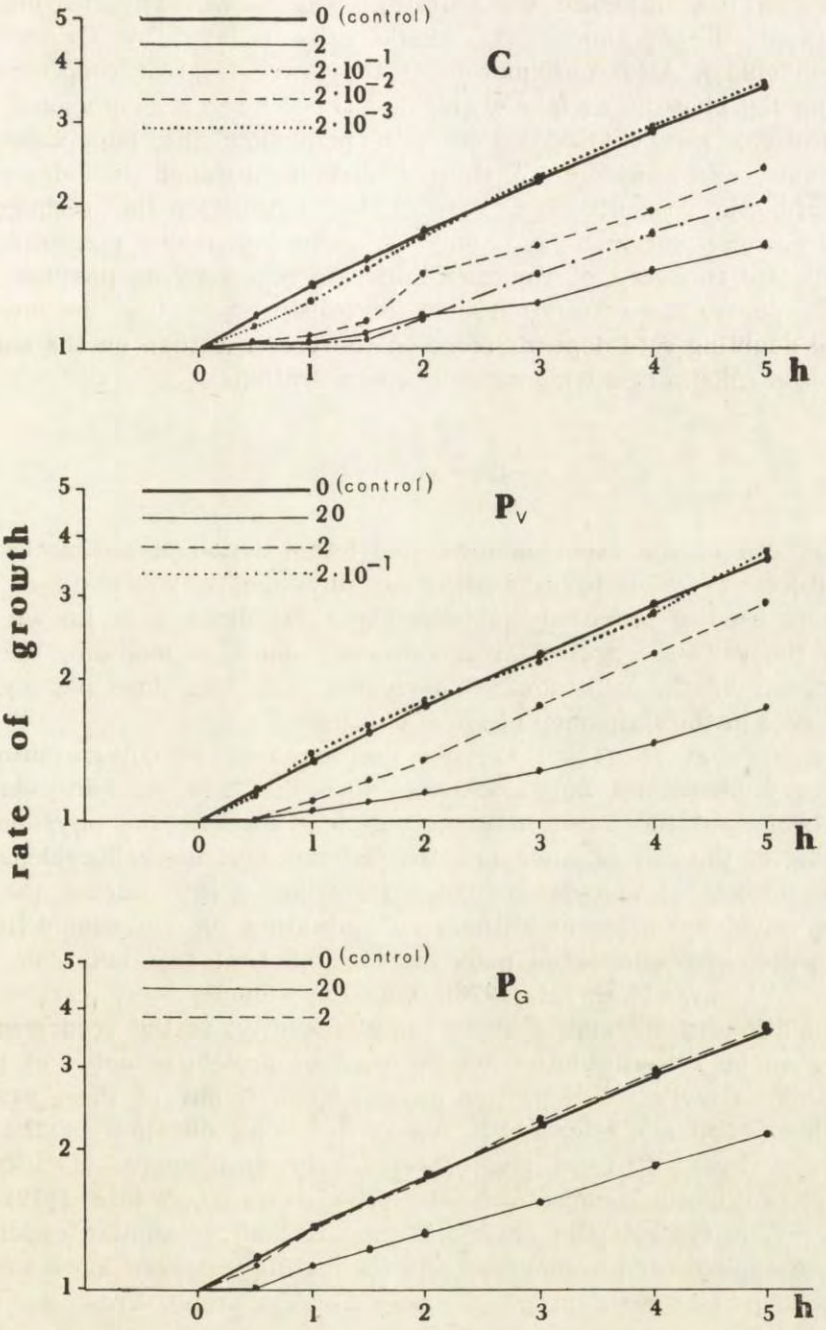

Fig. 1. The influence of different concentrations (in mM/l) of colistin (C), penicillin V (P_V) and penicillin G (P_G) on cell multiplication in exponentially growing mass cultures of *Tetrahymena pyriformis* during 5 h of experiment. Abscissa — time in hours after treatment, ordinate — rate of cell number growth

length on the antibiotic concentration was shown. This feature was confirmed in experiments on single cells isolated by Frankel's method (1963). Also confirmation of the lower level of concentrations causing lag phase (Szablewski and Oleszczak — personal communication) was obtained. For both penicillins the lag phase was the same, and penicillin V showed that its duration is independent on antibiotic concentration. For all the antibiotics the recovery of doubling was obtained, but only in lower penicillin concentrations a complete recovery of the doubling rate seems to be possible after the lag phase. The results obtained for colistin show, that the lowering of the doubling rate depends more on the presence than on the concentration of colistin in a wide range of concentrations.

Discussion

The aim of the experiment on the lethal action of antibiotics was to subject to their toxic action the physiologically equalized cells, what is usually ommited in toxicological studies. It is known that when the cells are grown in a constant volume of medium, the rate of growth of the cell number decreases with the time passing, till about zero in the stationary phase of culture.

Renter et al. (1980) showed that entering of culture into the stationary phase did not block the possibilities of cell division but only prolonged the duration of cell cycle. However, the physiological blocking of the cell to enter into the "S" phase of the cell cycle is one of the effects of starvation. This "transition point" during the cell life is called by different authors an "initiation" or "division/differentiation-decision" and takes place during the first two hours of starvation (Allewell et al. 1976). Our experiments were carried out in two different starving systems simultaneously, as the occurrence of synergism in Tris-antibiotics arrangement or protective acton of phosphate ions (Dryl's solution) was apprehended, if one of these systems had been arbitraly selected. It seems that long duration of the test (20 h) protected it from possible errors in consequence of different rate of antibiotic penetration into cells (Kuryłowicz 1979) and moreover, it controls the antibiotic toxicities under similar conditions to those applied for phagocytosis and for mating tests, see Rebandel and Karpińska (in prep.), Rebandel (in prep.). From the comparison of reactivity of non-initiated and initiated cells it follows (data from large number of papers) that exceeding of the above mentioned "transition point" in the cell life results in a considerable li-

mitation of its anabolic potencies. In this way it is assumed that the comparison of initiated and non-initiated stationary cell responses to the some chemical agents can be considered as a "screening procedure" useful in the preliminary differentiation of the character of the unknown agent action. It seems that the obtained results confirm the above mentioned assumption and, in this way, it can be further assumed that the mechanism of the colistin and penicillin toxic actions against *Tetrahymena pyriformis* is different.

The occurrence of the lag phase immediately after treatment with antibiotics suggests that the cell membrane is probably the seat of their action in tetrahymena too. The differences concerning the duration of the lag phase and its dependence upon concentration as well as rate of doubling after the recovery of the division process (on the basis of estimation of the approximative "doubling" between the 3rd and 5th h of experiment) confirm the above mentioned thesis about a different mechanisms of action of colistin and penicillins.

The toxicity of colistin was about 100 times higher than that of penicillins excluding the PPYS in the first type of experiment which seems to confirm the justification of assumption that colistin acts specifically while penicillins acts non-specifically against tetrahymena as model-animal cells in pharmacological sense.

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Cephaline Gregarine (*Protozoa*: *Sporozoa*) *Ramicephalus olivacus* sp. n. Parasite of an Odonate *Ceriagrion olivacum* Laidlow from India

Received on 28 August 1980

Synopsis. The cephaline gregarine (*Protozoa*: *Sporozoa*) *Ramicephalus olivacus* sp. n. has been described from the midgut of an odonate *Ceriagrion olivacum* Laidlow from West Bengal. It has the ratios of LP : TL = 1:6.13 and WP : LD = 1:0.96.

Obata (1953) instituted a new genus *Ramicephalus* to accommodate a cephaline gregarine, *Ramicephalus ozakii* from an coleopteran insect *Chlaenius inops* Chaudoir from Japan. The genus is characterized by (1) epimerite dish-like, with many upwardly directed dendroidal processes, arranged at the periphery (2) sporadin solitary, (3) cyst dehisces by simple rupture and (4) spores biconical, with rows of polar and equatorial spines, the latter being six in number. Since then, Théodoridès (1961), Tuzet et al. (1968) and Théodoridès et al. (1972, 1975, 1976) have added nine more species under the genus *Ramicephalus* Obata, 1953 from various insects.

The present communication reports a cephaline gregarine under *Ramicephalus* Obata, from the midgut of an odonate, *Ceriagrion olivacum* Laidlow from West Bengal. It is described hereunder as new species as it differs from all the known species of the genus.

Material and Methods

The insects were brought alive to the laboratory and their alimentary canals were dissected out in 0.5% saline water. Smears of infected midguts were made

on grease-free slides and fixed in Schaudinn's fixative. The highly infected midguts were fixed in Bouin's fluid and 5.0 μm thick serial paraffin sections were made to study intracellular development of the parasite. Both smears and sections were stained subsequently with iron alum haematoxylin method. The development of the gametocysts was observed after placing them in cavity slides and keeping them in moist chamber. The spores were examined with Lugol's iodine solution. The ratios used in this paper are the ratio of the length of protomerite to total length and the ratio of the width of protomerite to the width of deutomerite. All figures have been drawn with the aid of Camera lucida.

Observations

Ramicephalus olivacus sp. n.

Structure of the Trophozoite

The earliest stage obtained from the midgut smears is a three segmented trophozoite (Fig. 1 *1*) having oval to spherical epimerite, hemispherical protomerite and fusiform deutomerite with an ovoidal nucleus in the centre. The epimerite is somewhat elevated at the centre and is provided with fifteen to twenty short but stout spines in the periphery. The youngest trophozoite measures 58.5 μm in length and 23.1 μm in width in which the epimerite is almost spherical measuring 21.0 μm in diameter. The trophozoites when fully grown are elongated in shape (Fig. 1 *2*) and measure 323.4 μm in length and 101.6 μm in width in the average. The epimerite at this stage is a cylindroglobular body, differentiated into an upper hyaline, elevated, circular disc and a lower cylindrical longitudinally striated part. The disc is provided with fifteen to twenty sharply pointed spines arranged around its periphery (Fig. 1 *3*). In some well-preserved specimens, the ramification of the spines are nicely observed. Each of the spines is bifurcated at its middle (Fig. 1 *4*). The epimerite penetrates deep into the host gut epithelial cells and thus the gregarine established itself firmly there with the help of these ramified processes (Fig. 1 *10*). A very short neck connects the epimerite with the protomerite. The protomerite is almost hemispherical and is broader than long. The deutomerite is cylindrical with a rounded proximity. An ovoidal nucleus is situated at its anterior portion, immediately behind the septum.

Structure of the Sporadin

Sporadins are characteristically solitary measuring 542.0 μm in length and 136.8 μm in width in the average. The features of the

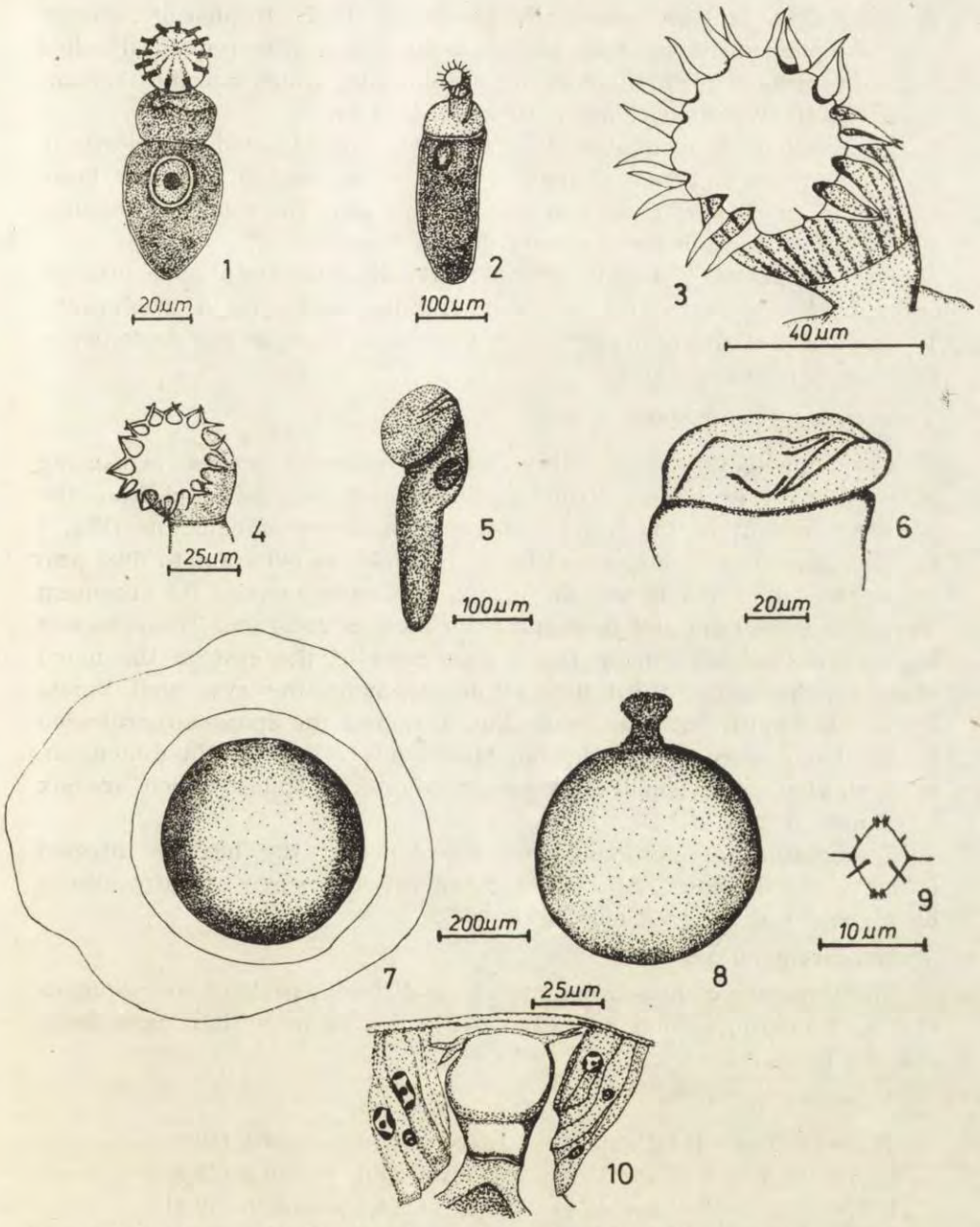

Fig. 1 1–10. Camera lucida drawings of *Ramicephalus olivacus* sp. n. *1* — An young trophozoite, *2* — A fully grown trophozoite, *3* — An epimerite — enlarged, *4* — An epimerite showing bifurcated nature of its spines, *5* — A fully grown sporadin, *6* — The protomerite of sporadin showing its sucker-like nature, *7* — Gametocyst with two layers of ectocyst, *8* — Gametocyst showing simple rupture of its wall, *9* — Biconical spore with polar and equatorial spines, *10* — Section through midgut showing attachment of epimerite with midgut epithelium

sporadin (Fig. 1 5) are essentially similar as in the trophozoite, except that an epimerite is wanting. Occasionally it is seen to remain attached with the midgut epithelium by its protomerite, which becomes rectangular and uneven so as to act as sucker (Fig. 1 6).

The nucleus is ovoidal and is almost always located anteriorly in the deutomerite. It has a distinct nuclear membrane and one large to several small karyosomes in the nucleoplasm. The nucleus measures 40.6 μm × 27.1 μm in dimension in the average.

The cytoplasm is highly granulated and is differentiated into an outer hyaline epicyte and an inner granular endocyte in trophozoite. The granules of the protomerite are fine while those of the deutomerite are coarser in the sporadin.

Gametocyst and Spore

The gametocysts are milky white, spherical bodies measuring 369.0 μm to 477.0 μm in diameter. The gametocyst, collected from the posterior midgut of the host, possesses two layers of ectocysts (Fig. 1 6). The inner one is almost uniform in thickness (40.0 μm to 60.0 μm) while the outer one is not uniformly thick everywhere; its minimum thickness is 69.0 μm and maximum thickness is 260.0 μm. The ectocysts layers are shed off during the development of the cyst in the moist chamber. After the third day of development, the cyst wall bursts by simple rupture at one point (Fig. 1 8) and the spores are released freely. Each spore is spindle-shaped, 6.0 μm × 4.5 μm in dimension, with shorter polar spines and longer equatorial spines which are six in number (Fig. 1 9).

Examination of numerous serial sections of the heavily infected midguts of the hosts, has failed to reveal any stage of intracellular development of the gregarine.

Measurements (in μm):

The summary of measurements of the different parts of 30 specimens of the trophozoites and sporadins with the mean within parenthesis is given below:

Trophozoite

TL = 58.8 — 411.6 (212.9), LNc = 12.6 — 29.4 (19.8),
LE = 16.8 — 46.2 (28.8), WE = 21.0 — 46.2 (32.3);
LP = 8.8 — 84.0 (41.4), WP = 14.7 — 138.6 (69.9),
LD = 29.4 — 273.0 (134.8), WD = 23.1 — 121.8 (70.2),

Sporadin

TL = 112.0 — 1024.0 (542.0),
LP = 32.0 — 240.0 (90.8), WP = 40.0 — 336.0 (134.4),

LD = 80.0 — 912.0 (451.2), WD = 48.0 — 384.0 (128,8),
LN = 20.0 — 80.0 (40.6), WN = 16.0 — 40.0 (27.1),
LP : TL = 1 : 6.13, WP : WD = 1 : 0.96.

Details of measurements of the different parts of 30 specimens are given in Table 1.

Seasonal Intensity

The insects were collected throughout the period of investigation. The parasites are mostly found during the months of September–October. Altogether, 16.0% of the host insects were found to be infected with the gregarine in their midgut.

Material

Holotype — on slide No. Od_6^{01}–6, prepared from the smears of the midgut content of the odonate, *Ceriagrion olivacum* Laidlow, collected on 28 May, 1978 at Hooghly, West Bengal, India and deposited at the Department of Zoology, University of Kalyani, Kalyani; Paratype-many, on the above numbered slide and on other slides as well, other particulars are the same as for holotype material.

Discussion

The cephaline gregarine under report possesses a complex epimerite with branched processes, the spindle-shaped spores with polar and equatorial spines and is rightly placed under the genus *Ramicephalus* Obata. The trophozoite of the gregarine resembles superficially with that of *Ramicephalus amphoriformis* Bush, 1928. But the highly complex epimerite of the present species having an anterior hyaline disc with fifteen to twenty bifurcated peripheral spines and posterior striated cylindrical part, a short neck, the measurements of various body parts and of spores and the odonate hosts are quite distinctive from the other species described so far of the genus *Ramicephalus* Obata. The gregarine is, therefore, considered to be a new species and is described here as *Ramicephalus olivacus* sp. n. The specific trivial name olivacus has been derived from the name of its host.

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Table 1

Details of measurements of the different parts of 30 specimens of *Ramicephalus olivacus* sp.n. (in μm):

Serial No.	TL	LE	LP	LD	LNc	LN	WE	WP	WD	WN	LP:TL	WP: WD
1	130.2	16.8	25.2	71.4	23.1	23.1	27.3	42.0	56.7	18.9	1:5.1	1:1.3
2	96.6	29.4	16.8	50.4	—	21.0	29.4	33.6	42.0	21.0	1:5.7	1:1.2
3	113.4	23.1	23.1	67.2	—	—	27.3	33.6	29.4	—	1:4.9	1:0.87
4	96.6	27.3	16.8	54.6	—	—	29.4	33.6	42.0	—	1:5.7	1:1.2
5	58.8	21.0	8.8	29.4	—	12.6	21.0	14.7	23.1	10.5	1:6.7	1:1.6
6	336.0	46.2	71.4	205.8	25.2	—	46.2	92.4	92.4	—	1:4.7	1:1.0
7	226.8	42.0	37.8	147.0	—	42.0	46.2	105.0	96.6	25.2	1:6.0	1:0.9
8	411.6	42.0	84.0	273.0	29.4	46.2	33.6	238.6	121.8	33 6	1:4.9	1:0.87
9	352.8	33.6	71.4	231.0	16.8	42.0	37.8	109.2	100.8	21.0	1:4.9	1:0.9
10	306.6	29.4	58.8	218.4	—	37.8	29.4	96.6	96.6	21.0	1:5,2	1:1.0
11	344.0	—	48.0	296.0	—	48.0	—	48.0	64.0	24.0	1:7.2	1:1.3
12	112.0	—	32.0	80.0	—	20.0	—	40.0	48.0	16.0	1:3.5	1:1.2
13	240.0	—	48.0	192.0	—	32.0	—	80.0	72.0	20.0	1:5.0	1:0.9
14	464.0	—	64.0	400.0	—	32.0	—	64.0	56.0	24.0	1:7.2	1:0.87
15	400.0	—	64.0	336.0	—	32.0	—	64.0	64.0	24.0	1:6.2	1:1.0
16	448.0	—	56.0	392.0	—	40.0	—	48.0	48.0	24.0	1:8.0	1:1.0
17	416.0	—	72.0	344.0	—	40.0	—	80.0	72.0	20.0	1:5.7	1:0.9
18	864.0	—	208.1	656.0	—	64.0	—	256.0	224.0	48.0	1:4.1	1:0.87
19	896.0	—	240.0	656.0	—	80.0	—	256.0	208.0	48.0	1:3.7	1:0.8
20	672.0	—	80.0	592.0	—	48.0	—	144.0	144.0	32.0	1:8.4	1:1.0
21	912.0	—	112.0	800.0	—	48.0	—	240.0	256.0	40.0	1:8.1	1:1.06
22	944.0	—	144.0	800.0	—	48.0	—	176.0	176.0	32.0	1:6.5	1:1.0
23	960.0	—	128.0	832.0	—	40.0	—	208.0	192.0	40.0	1:7.5	1:0.9
24	560.0	—	64.0	496.0	—	40.0	—	96.0	128.0	28.0	1:8.7	1:1.3
25	288.0	—	64.0	224.0	—	36.0	—	96.0	80.0	24.0	1:4.5	1:0.8
26	560.0	—	128.0	432.0	—	64.0	—	256.0	352.0	32.0	1:4.4	1:1.37
27	1024.0	—	112.0	912.0	—	40.0	—	336.0	384.0	40.0	1:9.1	1:1.1
28	208.0	—	40.0	168.0	—	32.0	—	48.0	48.0	16.0	1:5.2	1:1.0
29	320.0	—	64.0	256.0	—	36.0	—	104.0	72.0	24.0	1:5.0	1:0.7
30	208.0	—	48.0	160.0	—	24.0	—	48.0	48.0	24.0	1:4.3	1:1.0

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ACTA PROTOZOOLOGICA

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Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani, Nadia, West Bengal, India

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Cephaline Gregarine *Tetractinospora victoris* gen. n., sp. n., Parasite of an Odonate *Ceriagrion coromandelianum* (Fabricius) from India

Received on 10 November 1980

Synopsis. The morphology and life history of the cephaline gregarine ***Tetractinospora victoris*** gen. n. sp. n. have been described from an odonate, ***Ceriagrion coromandelianum*** (Fabricius) from West Bengal, India. The new genus *Tetractinospora* is characterized by the globular epimerite with several vertical lamellae and biconical spores with four short sharp and stout spines, two at each pole. A comparative study of the different genera including the new one under the subfamily *Acanthosporinae* Léger, 1892 emend Grassé, 1953 has also been made to establish the distinctiveness of the new genus.

The insects are known today as the most prolific hosts of cephaline gregarines. But only a few of them have been described from the odonate insects by Léger (1892), Crawley (1903), Ellis (1914), Kamm (1922), Hoside (1953), Stein (1960), Desportes (1963), Boudoin (1967), Geus (1969), Sarkar and Chakravarty (1969), Devdhar and Despande (1971), Nazeer Ahmed and Narasimhamurti (1979) and Sarkar and Haldar (1980).

While examining the midgut contents of various arthropod hosts of West Bengal, a cephaline gregarine has been obtained from an odonate insect *Ceriagrion coromandelianum* (Fabricius) and is described hereunder as *Tetractinospora victoris* gen. n. sp. n. for several of its unique features.

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Materials and Method

The insects were brought alive to the laboratory, the alimentary canals were dissected out with 0.5% saline water on clean grease-free slides under the dissecting binocular and were examined for the protozoan parasites. The rest of the methods employed here, has been adopted from **Haldar and Sarkar** (1980). The abbreviations used in this paper are, LD — length of deutomerite, LE — length of epimerite, LN — length of nucleus, LNc — length of neck, LP — length of protomerite, TL — total length, WD — width of deutomerite, WE — width of epimerite, WN — width of nucleus and WP — width of protomerite. The ratios used are the ratio of the length of protomerite to total length and the ratio of the width of protomerite to the width of deutomerite (LP : TL and WP : WD respectively). All figures have been drawn with the aid of Camera lucida.

Observations

Tetractinospora victoris gen. n. sp. n.

Host: *Ceriagrion coromandelianum* (Fabricius)

The trophozoite (Fig. 1 *1*) obtained from the smears of the midgut contents of the host measures 250.3 μm in length and 49.8 μm in width in the average having a globular epimerite with a short neck, a rectangular protomerite and an elongated conical deutomerite. The epimerite (Fig. 1 *2*) measures 20.0 μm × 23.2 μm in dimension in the average and is beset with more than eight (about sixteen) bent, hyaline plates arranged longitudinally so that it gives a laminate appearance. The anterior end of the epimerite is round while the posterior end is surrounded by the sharp posterior edges of the laminate hyaline plates. The epimerite is connected with the protomerite by a short neck, 6.3 μm long in the average. The protomerite is almost rectangular and is typically broader than long. The deutomerite is elongated having an ellipsoidal nucleus and gradually narrows down to a blunt end. The septum between the protomerite and deutomerite is somewhat lenticular.

The sporadins are characteristically solitary and measure 349.5 μm in length and 74.4 μm in width in the average. In young sporadins, the protomerite is somewhat dome-shaped (Fig. 1 *3*), but in older forms this is almost rectangular (Fig. 1 *4*). The deutomerite is elongated with a posterior ovoidal nucleus and terminates in a sharply pointed end.

The cytoplasm is granulated and uniformly distributed in both protomerite and deutomerite. It is differentiated into an outer hyaline

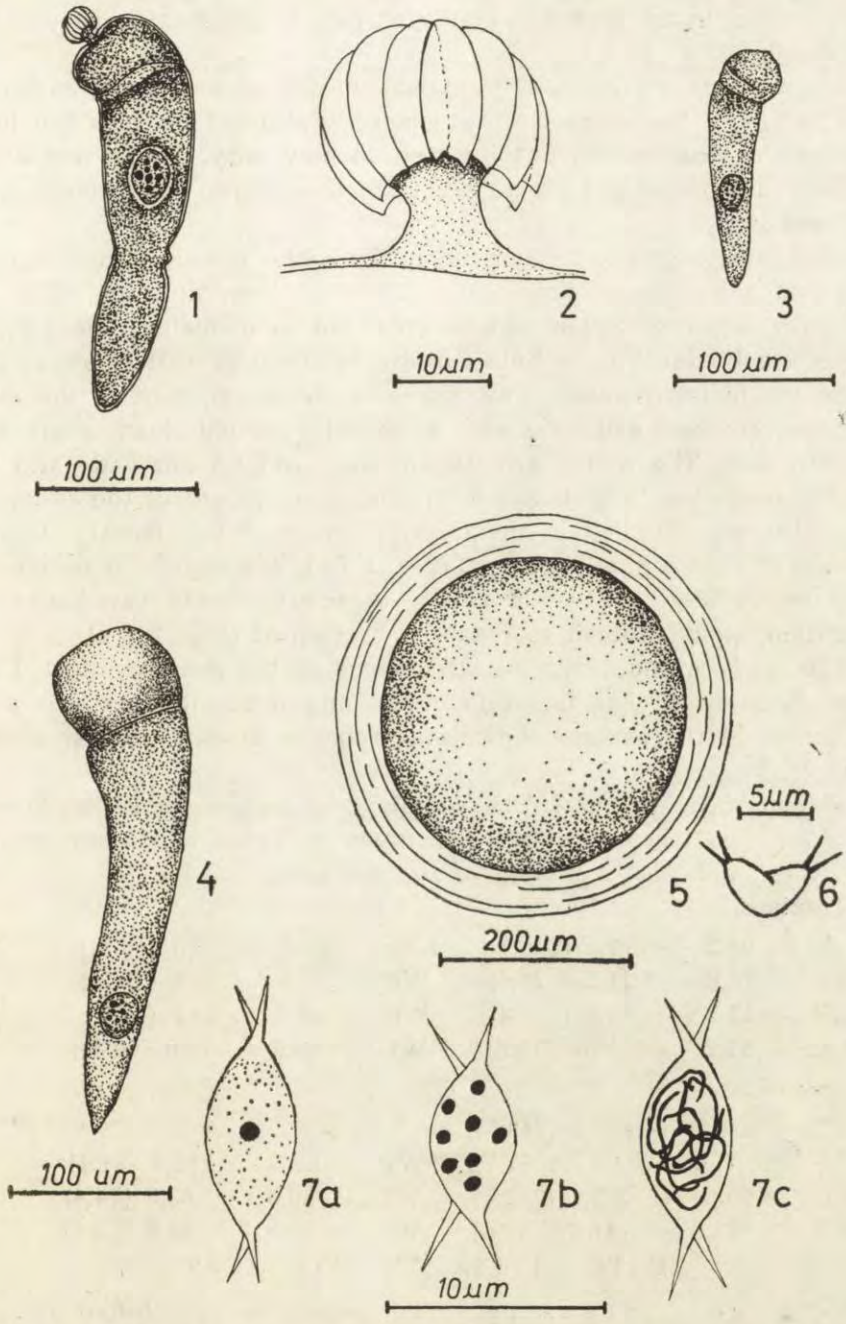

Fig. 1. 1–7 — Camera lucida drawings of *Tetractinospora victoris* gen. n. sp. n. *1* — A fully grown trophozoite, *2* — The epimerite — enlarged, *3* — An young sporadin, *4* — A fully grown sporadin, *5* — Spherical gametocyst with thick layer of ectocyst, *6* — Biconical spore showing its bent in the middle and polar spines, *7* — Spores with various developmental stages of sporozoites, *7 a* — Uninucleate spore, *7 b* — Octonucleate spore, *7 c* — Spore with filiform sporozoites

epicyte and inner granular endocyte but longitudinal myonemes are not distinct.

The nucleus is ellipsoidal to ovoidal in outline measuring 36.0 μm × × 23.0 μm in the average. It is generally situated towards the hinder part of the deutomerite but its position may vary. There is a distinct nuclear membrane and six to eight small spherical karyosomes in the nucleoplasm.

The gametocysts are spherical, milky white bodies with a thin cyst wall and a hyaline ectocyst with almost uniform thickness (Fig. 1 5). The cyst measures 300.00 μm to 350.0 μm in diameter. The refractile spores are liberated by simple rupture of the cyst wall at about 120 h inside the moist chamber. The spores are biconical, bent in the middle and are provided with one pair of sharply pointed short, stout spines at each pole. The spores are 9.0 μm long and 4.5 μm wide and their spines are 4.5 μm long (Fig. 1 6, 7). The development of the sporozoites have also been studied in the moist chamber. When freshly liberated, the spores have a single nucleus (Fig. 1 7 *a*). Afterwards, it divides into eight nuclei (Fig. 1 7 *b*) and finally at about seventy two hours after liberation, eight filiform sporozoites are formed (Fig. 1 7 *c*).

The study of numerous serial sections of the heavily infected midguts of the hosts, has failed to obtain any intracellular stages of development of the parasite indicating probably its extracellular development.

Measurements (in μm). The summary of measurements of 20 specimens are given below; details are given in Table 1. Measurements in parenthesis indicate an average of 20 specimens:

Trophozoite

TL = 93.2 — 312.5 (250.3), LNc = 4.2 — 12.5 (6.3);
LE = 18.0 — 21.2 (20.0); WE = 20.8 — 27.5 (23.2);
LP = 12.7 — 45.8 (35.4); WP = 16.9 — 54.1 (43.0);
LD — 57.2 — 233.3 (118.6); WD = 23.3 — 72.0 (49.8);

Sporadin

TL = 226.7 — 466.7 (349.5),
LP = 50.0 — 83.3 (67.7), WP = 58.3 — 112.5 (81.4);
LD = 208.4 — 383.4 (281.0), WD = 50.0 — 83.3 (74.4);
LN = 33.3 — 41.6 (36.0), WN = 20.8 — 29.6 (23.0);
LP : TL = 1 : 5.2, WP : WD = 1 : 0.9

Seasonal intensity. The insects were collected from the Kalyani University campus during the months of April to October, 1978. Altogether, 35.0% of the insects were found to be infected in their midgut with this gregarine.

Table 1

Details of measurements of the different parts of 20 specimens of *Tetractinospora victoris* sp. n. (in μm)

Serial No.	TL	LE	LP	LD	LNc	LN	WE	WP	WD	WN	LP:TL	WP: WD
1	312.5	20.8	45.8	233.3	12.5	33.3	20.8	54.1	54.1	25.0	1:6.8	1:1.0
2	283.3	21.2	42.4	220.4	4.3	38.1	23.3	50.8	53.0	21.2	1:6.7	1:1.04
3	256.5	18.0	33.9	199.3	5.3	—	25.4	42.4	46.6	—	1:7.6	1:1.09
4	93.2	19.0	12.7	57.2	4.2	—	19.0	16.9	23.3	—	1:7.3	1:1.38
5	301.0	21.2	42.4	233.2	4.2	38.1	27.5	50.8	72.0	29.6	1:7.1	1:1.42
6	333.4		58.3	275.1		41.6		75.0	75.0	20.8	1:5.72	1:1.0
7	333.4		66.6	266.8		33.3		75.0	66.6	25.0	1:5.01	1:0.89
8	350.0		66.6	283.4		33.3		83.3	75.0	25.0	1:5.2	1:0.90
9	383.4		75.0	308.4		33.3		87.5	79.1	25.0	1:5.1	1:0.90
10	300.0		83.3	216.7		33.3		58.3	58.3	25.0	1:3.6	1:1.0
11	366.7		75.0	261.7		33.3		91.6	83.3	25.0	1:4.89	1:0.91
12	350.0		66.6	283.4		37.5		112.5	83.3	20.8	1:5.2	1:0.74
13	283.03		66.6	216.7		37.5		83.3	75.0	20.8	1:4.2	1:0.90
14	350.0		66.6	283.4		37.5		75.0	66.6	20.8	1:5.2	1:0.89
15	366.7		66.6	300.1		37.5		100.0	79.1	20.8	1:5.5	1:0.79
16	383.4		50.0	333.4		37.5		79.0	83.3	25.0	1:7.67	1:1.05
17	333.4		58,3	275.1		37.5		83.3	83.3	20.8	1:5.7	1:1.0
18	366.7		75.0	291.7		33.3		75.0	75.0	20.8	1:4.89	1:1.0
19	266.7		58.3	208.4		33.3		58.3	50.0	20.8	1:4.57	1:0.86
20	466.7		83.3	383.4		41.6		83.3	83.3	25.0	1:5.6	1:1.0

LP:TL = 1:3.6 – 7.67 (1:5.2); WP: WD = 1:0.74 – 1.42 (1:0.9)

Table 2

A comparative study of the different genera under the subfamily *Acanthosporinae* Léger, 1892 emend Grassé, 1953

Genera	Characters	
	Epimerite	Spore
(1) *Acanthospora* Léger, 1892	A simple conical knob;	Biconical with one row of polar and one row of equatorial spines
(2) *Corycella* Léger, 1892	Globular with eight large curved hooks,	Biconical with one row of polar spines
(3) *Ancyrophora* Léger, 1892	Globular with five to twelve backwardly directed digitiform processes	Biconical with one row of polar and one row of equatorial spines
(4) *Cometoides* Labbé, 1899	Globular with six to eight long, slender filaments directed upwards,	Cylindro-biconical with one row of polar and two rows of equatorial spine
(5) *Prismatophora* Ellis, 1914	Sub-globular with eight lateral recurved hooks,	Haxagonal with one row of spines at each pole
(6) *Tetraedospora* Tschudovskaia, 1928	Flatten and disc-like with fourteen to sixteen hooks (crochets),	Tetrahedral and elongate with limits of the faces bearing a row of spines
(7) *Ramicephalus* Obata, 1953	Dish-like, with many upwardly directed dendroidal processes,	Biconical with one row of polar and one row of equatorial spines
(8) *Dinematospora* Tuzet and Ormières, 1954	Hemispherical, attached to the protomerite by a chromophilic ring persisting upto the time of syzygy	Oval, provided with long filaments at each pole
(9) *Spinispora* Baudoin, 1967	Not known,	Fusiform, covered by spines all over the surface
(10) *Quadruspinospora* Sarkar and Chakravarty, 1969 emend Haldar and Chakraborty, 1975	Subspherical, with variable number of stumpy digitiform processes,	Oval spores with four long spines, two at each pole
(11) *Doliospora* Ormières and Baudoin, 1969	Simple globular,	Barrel-shaped, asymmetrical, with two lateral spines on opposite sides
(12) *Rhizionella* Baudoin, 1971	Button-like, with eight long rhizoides on the periphery,	Biconical, each pole ends into a pointed prolongation and six short equatorial spines
(13) *Contospora* Devdhar and Amoji, 1978	Conical knob dentated at the base with series (about 20) of vertical lamellae,	Cylindrical with pointed ends with a tuft of spines at each end
(14) *Echinospora* Amoji and Devdhar, 1979	Simple, globular or spherical knob,	Biconical with a row of eight to ten slender polar spines at each pole, released in chains of two to three or more
(15) *Tetractinospora* new genus	Globular with many (more than eight) vertical curved plates or denticles arranged in the form of lamellae, placed on a short neck,	Large, biconical, bent in the middle, with four sharp and stout spines, two at each pole

Material. Holotype — on slide No. Od_6 C–8, prepared from the midgut contents of *Ceriagrion coromandelianum* (Fabricius), collected on 13 September, 1978 and deposited at the Department of Zoology, Kalyani University; Paratype — many, other particulars are the same as for holotype material.

Discussion. The cephaline gregarine under report possesses solitary sporadin, globular epimerite with laminate vertical plates, short neck and biconical spores with four sharp, stout spines, two at each pole. The presence of spines in the spore justifies its inclusion under the subfamily *Acanthosporinae* Léger emend, Grassé of the family *Actinocephalidae* Léger, 1892. However, the detail structure of the epimerite and the spore of the gregarine together, are not found in any of the existing genera of the subfamily *Acanthosporinae*. A new genus *Tetractinospora* is, therefore, proposed to accommodate this gregarine and the name *Tetractinospora victoris* gen. n. sp. n. is given to it. A comparative study of the different genera under the subfamily *Acanthosporinae* Léger emend Grassé with the proposed genus *Tetractinospora* gen. n. has been made in Table 2.

The specific trival name *victoris* is given after the eminent Protozoologist Dr. Victor Sprague of the Chesapeake Biological Laboratory of Maryland, U.S.A.

Diagnosis: The diagnostic features of the genus *Tetractinospora* gen. n. are summarized below:

(1) epimerite globular with several (about sixteen) laminate vertical plates, placed on a short neck,

(2) sporadins solitary,

(3) cyst dehisces by simple rupture of its wall,

(4) spores biconical, bent in the middle, with four sharp stout spines, two at each pole.

Type species: *Tetractinospora victoris* gen. n. sp. n.
Host: *Ceriagrion coromandelianum* (Fabricius)
Site of infection: Midgut
Locality: Kalyani, West Bengal, India.

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ACTA PROTOZOOLOGICA

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Glugea nemipteri sp. n. and *Nosema bengalis* sp. n., Two New Microsporidia of *Nemipterus japonicus* in India

Received on 28 September 1980

Synopsis. Two microsporidia, *Glugea nemipteri* sp. n. and *Nosema bengalis* sp. n. are described from muscles, gonads and liver, and gills respectively of *Nemipterus japonicus* collected in the Gulf of Bengal. Infections are distributed in separate areas and do not interfere. Number of infected fishes decreases with the age of the hosts.

The microsporidian parasites of fishes, especially of marine species have received little attention from the point of view of their ecology and geographical distribution. With a limited array of morphological markers and lack of experimental cross infections, their diagnostics is rather difficult. This is especially the case with the members of the genus *Glugea*, the most common pathogens of fishes. In course of a study of the parasites of the fishes of the Waltair coast of the Gulf of Bengal, India, we came across two new microsporidian parasites of the fish *Nemipterus japonicus*, differing in morphology and the infected tissues. The infections are described in the following account.

Material and Methods

Fishes collected from the catches of the Offshore Fishing Station at Vishakapatnam, Lawson's Bay colony and Bheemunipatnam (about 20 km North of Vishakapatnam) were immediately brought to the laboratory, dissected, and the different organs examined for infection using either a head-band magnifier or a binocular dissecting microscope. Cysts of the parasites were fixed along with the host tissue in aqueous Bouin's fluid or Carnoy's fluid for preparation of paraffin blocks. Sections were cut at 8 μm thickness and stained with

Heidenhain's iron hematoxylin and wet Giemsa. After isolating the xenomas from the host tissue, smears were prepared, air dried, fixed in methyl alcohol and stained with Giemsa. Parts of such smears were hydrolysed in 10% HCl hot at boiling for 1 s, washed and restained shortly (1–2 min) with Giemsa for demonstration of the nuclei. Smears were also wet fixed in Schaudin's or alcoholic Bouin's fluid and stained with azo-carmine or iron hematoxylin. Carnoy fixed material was used for Feulgen preparations.

Incidence of Both Infections

Two different infections were recognized, differing in their localization in the host body. This with large spores was mainly in the gills, the other, microsporal, in visceral muscles, gonads and the liver (Table 1) and they varied in distribution in the collecting area. In the

Table 1

Origin of fish and distribution of infection

Locality	Pathogen	Number of fish coll.	Number of fish infected	Site of infection	Cyst size
Offshore Fishing station	*Nosema bengalis*	164	24 14.6%	gills	0.5–0.8 mm
Lawson's Bay	*Glugea nemipteri*	82	9 11%	muscles gonads liver	8–12 mm
Bheeminipatnam	—	58	— —	—	—

different localities one or the other infection were present, without interfering and mixed infections. It shows to some extent an existing focus-like distribution of the diseases in local populations.

The infection rate was high in smaller fish, with more than 16% infected fishes below 12 cm and dropped down in the bigger fish, as

Table 2

Size of fish *N. japonicus* with microsporidia

Size of fish groups in cm	No. of fish examined	No. of fish infected	Percentage of infection
7.5–10	49	8	16.33
10–12.5	112	18	16.1
12.5–15	93	3	3.2
15–17.5	32	1	3.1
17.5–20	18	—	—

summarized in Table 2. Both the percentage infection and availability of hosts was maximum during March–April. In the studied populations there was an evident elimination of the cysts in older fish. It probably was the result of bursting the cysts and regeneration of the tissues. Another reason could be a mortality in infected categories, but there was no evidence of severe damage due to the infections.

Description of the Parasites

Two infections were recognized in the infected fishes. One with a thin-walled cyst not bigger than 1 mm in the central part of the gills, with spores not longer than 3.5 μm, and a second with thick-walled 8 to 12 mm broad cysts in internal organs, mainly the muscles, gonads and the liver, spores longer than 5 μm.

Glugea nemipteri sp. n. (Fig. 1, a–j, Pl. I 1–4, II 1–3)

Host: *Nemipterus japonicus*, muscles, gonads and liver.
Locality: Lawson's Bay near Waltair, Gulf of Bengal, India.

The cysts containing stages of the parasite were attached and inserted into internal organs, especially muscles, gonads and the liver. Cysts were gray, oval to spherical, closed in a resistant outer elastic cyst wall, 30 to 50 μm thick. The cysts represent typical xenomas, one single host cell each in an envelope of connective tissue. The outer layer of connective tissue (Ct in Fig. 1 a) is 20–30 μm thick and contains nuclei of cells forming this layer, sometimes also erratic stages of the microsporidian. An internal layer of membranous structures (Fig. 1 a — M. Pl. I 1, 2), 15–20 μm thick covers the xenocyte with the microsporidian. The xenocyte is a typical hypertrophic host macrophage with nuclei divided into many fragments and distributed in the cytoplasm of the xenocyte beneath the membrane (Fig. 1 a–C, Pl. I 1, 3–N). In the studied material the development of the parasite was far in progress and most of the cytoplasm was vacuolated, with stages of the microsporidian concentrated in oval to spherical compartments lined with a rather persistent wall. The nuclei (N) of the xenocyte are squeezed among these compartments (Pl. I 3 c–v, 3 d–p) with sporoblasts usually close to the remains of nuclei. Only a fragmentary series of developmental stages was recognized. Uninucleate and binucleate schizonts grow into elongate cylinders with a series of nuclei in a zig-zag row. The nuclei form twins. Up to 12 nuclei were present in one cylinder 15–25 μm long and 6–9 μm broad. They are included

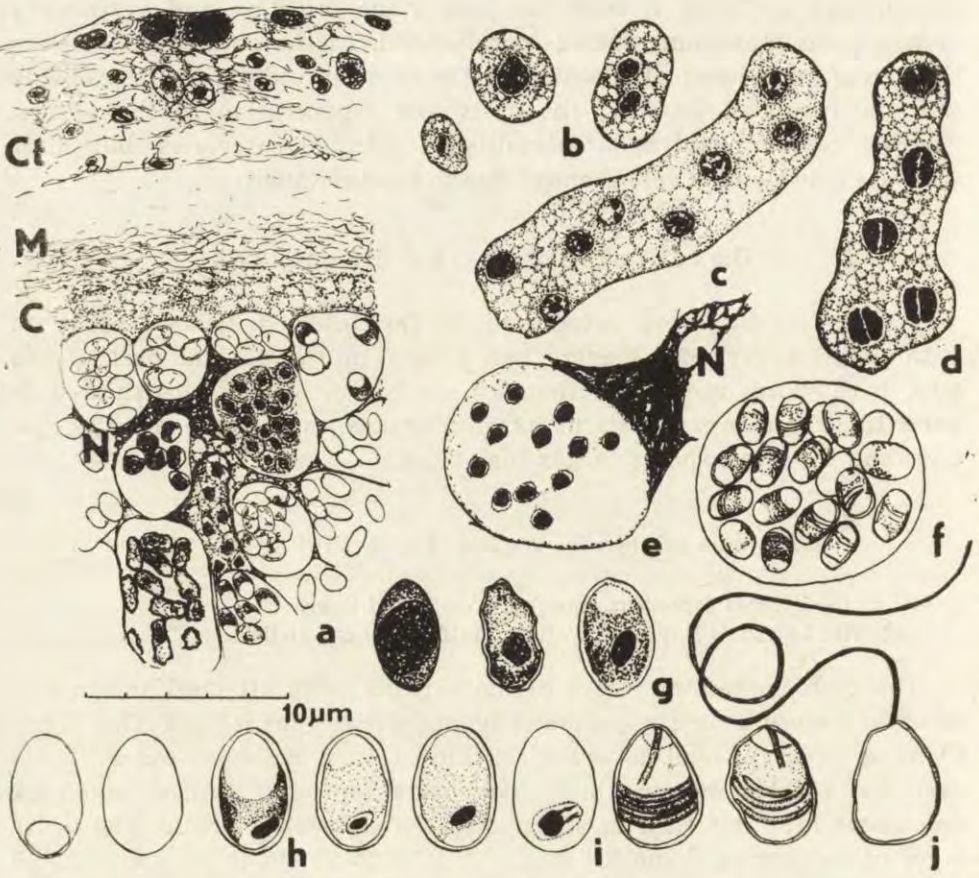

Fig. 1. *Glugea nemipteri*: a — Cross section of a cyst wall, (Ct — connective tissue, M — membranous layer, C — cytoplasm of xenocyte with its nuclei N), b — vegetative schizonts, c — cylindres, d — division of nuclei in cylindres, e — sporonts in a parasitophorous vacuole inserted in a host nucleus, N, f — mature spores in the vacuole, g — sporoblasts, h — mature spores, fresh and Giemsa stained on smear, with posterosome, i — nuclear staining of spores after HCl hydrolysis, j — coils of polar filament in fixed spores. Scale only for g–j

in the cytoplasm close to host nuclei without forming vacuoles first. Then they divide into fragments and single cells with 2 nuclei which belong to the series of autogamy with well visible chromosomes. The procedure could not be followed in details. Finally a group of sporonts is formed which all remain in a vacuole (parasitophorous vacuole, P — Pl. I 4) which has formed during the division of the cylinder. The group contains 16 or 32, sometimes 64 spherical stages with a dense spherical nucleus and a badly visible layer of cytoplasm. The vacuole is lined with a wall which makes the compartment for the spore for-

mation. The wall seems to result from an interaction between the host cell cytoplasm and the parasite. Oval sporoblasts and spores are formed in this envelope and remain in this compartment till maturation. Later the whole pack of spores is pushed to the central part of the cyst, some structures break and burst, others remain intact in the honeycomb-like structure. They are replaced by new sets of schizonts developing in the cortical cytoplasmic layer.

The spores are broadly oval, with broad blunt ends, with a vacuole in the posterior part of fresh spores. Stained with Giemsa they show a deep anterior vacuole with the polaroplast and a sporoplasm concentrated in the posterior part (Fig. 1, g, h, Pl. II 1, 2). A broad oval posterosome is stained sometimes in the posterior vacuole. With HCl-hydrolysis one single nucleus is stained in the posterior third of the spore. It is oval, oblique, sometimes broken by hydrolysis in two adjacent particles (Fig. 1 i, Pl. II 3). In section preparations stained with Giemsa the coiled polar filament is sometimes visible with four coils. On smears the polar filament is rarely extruded. Spore measurements on smears and in section preparations give dimensions 5.5–6 × 4.5–5 μm, most common are spores 5.9 by 4.5 μm. The polar filament where well extruded was uniformly thin and measured 40 to 60 μm.

This microsporidian belongs to the typical genus *Glugea* of *G. anomala* — type with a thick-walled cyst, the xenocyte with fragmented nuclei in the plasmatic layer and with spores in groups of 16 to 60. It differs from known species by its broad oval spores. We propose for this microsporidian the name *Glugea nemipteri* sp. n.

Nosema bengalis sp. n. (Fig. 2, a–i, Pl. II 4).

Host: *Nemipterus japonicus*, gills.
Locality: Visakhapatnam, Offshore Fishing Station, Gulf of Bengal, India.

This microsporidian was present in minute spherical gray cysts only 500 to 800 μm in diameter in the gills of young fish. The cyst wall was thin and continuing in the host tissue, bursting easily when isolation was attempted. The wall was only 2–3 μm thick, originating probably in the wall of the invaded host cell in the tissue. The cell content did not show details of the former host cell, there was no plasmatic layer with fragments of nuclei. Vegetative stages of the microsporidian were rare, only a few kidney shaped or broad oval binucleate sporonts were scattered in the spore mass. Sporoblasts were oval, mature spores piriform, with a narrow anterior end, a vacuole in the broader end. When fixed on smears or in section preparations, spores were piriform, with a pointed anterior end, a broad bulb in the second third of their length

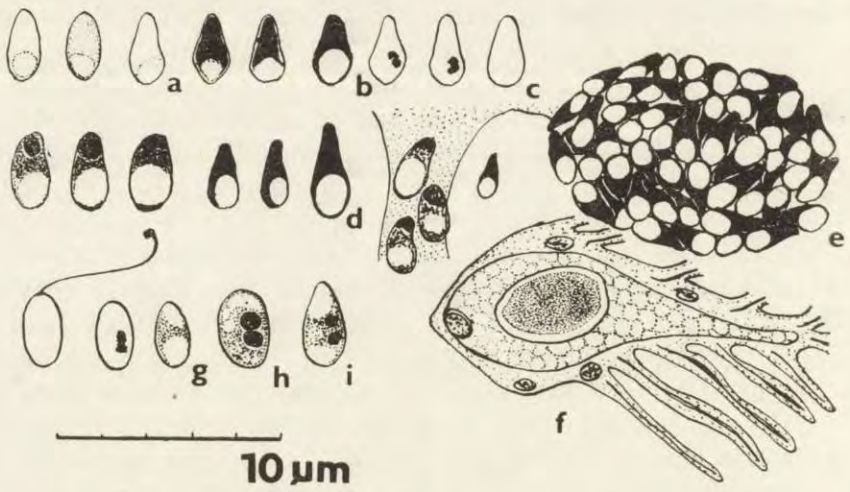

Fig. 2. *Nosema bengalis*: a — fresh spores, b — spores in sections with visible posterior vacuoles, Heidenhain's stain, c — nuclear staining of spores, d — spores on different smears, e — fragment of the spore mass, g — partially extruded filament on smear, h, i — sporoblasts on dry smears, f — gill with the cyst. Scale for all drawings except f

and a constricted posterior end. In stained specimen the great vacuole in the posterior pole was well visible. In HCl-treated spores a nuclear complex of two adjacent minute spherical nuclei was stained with Giemsa, without evidence of any other structure. The polar filament extruded after addition of a droplet of hydrogen peroxide was 60–80 µm long, in many cases a partly released polar filament was visible. Fresh spore dimensions were 3×2 µm and 3–3.5×1.5–1.7 µm in fixed smears. On smears spores were present in large, irregular groups without any sign of a pansporoblastic membrane. Due to lack of other developmental stages and the simple organization of the cyst with binucleate spores, the microsporidian is identified as a *Nosema*. It differs in its localization and the spore size from other microsporidia of the same genus in fishes. Therefore we propose for it the name *Nosema bengalis* sp. n.

Discussion

More than 20 species of *Glugea* and only some four species of *Nosema* are known from fishes and in many cases the morphology of their spores and cysts is very similar, with differing hosts and region of distribution. Such cases deserve registration and revision including

synonymization from time to time. Our *Glugea* with broad oval spores is quite isolated in the whole series with its spore size and shape and quite easy to distinguish. In our material we could not recognize finger-like disintegration of the sporogonial plasmodium at the beginning of the spore formation, which is well presented in the case of *G. atherinae* by Berrebi (1979). In our material there is a gap between the cylindres and free sporoblasts. From the point of view of zoogeography of the infection, the isolated, focus-like appearance of the infections on shore banks of the coast of Bengal is evident. With this species, as it is the case with each recently described member of the genus *Glugea* it is more and more evident that the definition of the genus has to be emendated in the way proposed by Weiser (1977). In contrary, the original formulation of Kudo (1924) requesting a bisporal sporogony, was not based on real observations on the type species, where twin spores are not present, and the one division of which two spores may result, is just one of several divisions of the sporont before formation of sporoblasts. The groups of spores sticking together in individual parasitophorous vacuoles of the xenocyte indicate the real number of divisions in sporogony which are five or six. A reaction to this irrealistic very schematic definition of the genus *Glugea* coinciding with *Perezia* and *Nosema* was the reason of the proposal of synonymy in the paper by Lom and Weiser (1969). At that time it was evident to the mentioned authors that a "paper" scheme and definition of the genus does not fit to the real nature of the genus.

The generic position of the second parasite is more complicated. The isolated, single, thin-walled cyst and the spores with two nuclei are not belonging to the genus *Glugea*. The cyst seems to be a migrating lymphocyte which was catched in a narrow passage due to its large size after development of the microsporidian. There is no spread of the infection in next tissues around. It is difficult to compare our case with "*Spraguea*", a genus which is mentioned by Sprague (1977) as being proposed by Weissenberg in 1976, but was not formulated at all in Weissenberg's paper and is mentioned only in a non-signed footnote of Sprague or Vavra on p. 216. The description was not formulated by Weissenberg in the final draft and could not be ascribed to him. The mentioned definition — "Formation of colonies of the parasite only at the cytoplasmic zone of the host cell where the invasion has originally started. Location of groups of multiple schizonts only at a medium layer of the colony" — fits in the early cycle of development of any *Glugea* and there is no other differentiating symptom given. Competting with *Nosema* in fishes is the genus

Ichthyosporidium Caullery and Mesnil, 1905 with binucleate spores, in a cyst with extensive ramifications and multicystic lesions. The cysts in our case are not ramified, are single, not multicystic. We therefore propose in our microsporidian to retain the generic name *Nosema* and compare again this material with mentioned other genera when more datails of development will be known of these parasites.

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EXPLANATION OF PLATES I–II

Pl. I. *Glugea nemipteri*

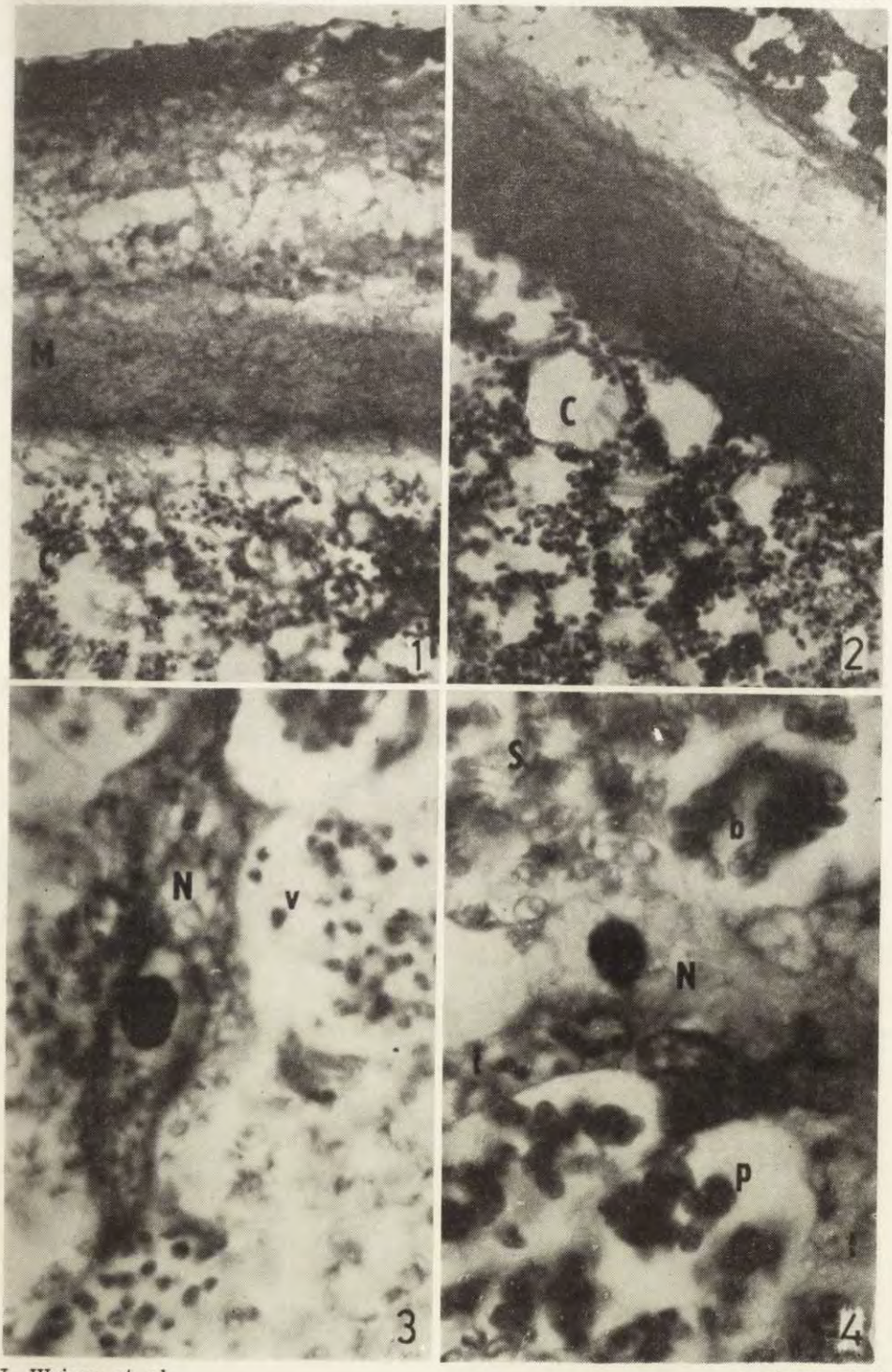
a: cross section of cyst wall (M — membranous layer, C — xenocyte)
b: cyst with spores in compartments (parasitophorous vacuoles)
c: host cell nucleus (N) with sporoblasts in parasitophorous vacuoles (v) inserted in the nucleus
d: section of cyst content with host nucleus (N), prosporoblasts (p), sporoblasts (b) and mature spores (S), in cytoplasm a cylinder (t)
Magnification — a, b — 450 ×, c, d — 1000 ×. Heidenhain's stain

Pl. II. *Glugea nemipteri*

a: mature spores (S) and prosporoblasts (p) dissolving the nucleus (N) of host cell
b: nucleus of xenocyte (N) invaded with binucleate stages (autogamets — a) and sporoblasts (v). Spores with visible nuclei
c: staining of nuclei with Giemsa after HCl-hydrolysis

Nosema bengalis

d: spores on smear
Magnification — a, b, d — 900 ×, c — 1400 ×



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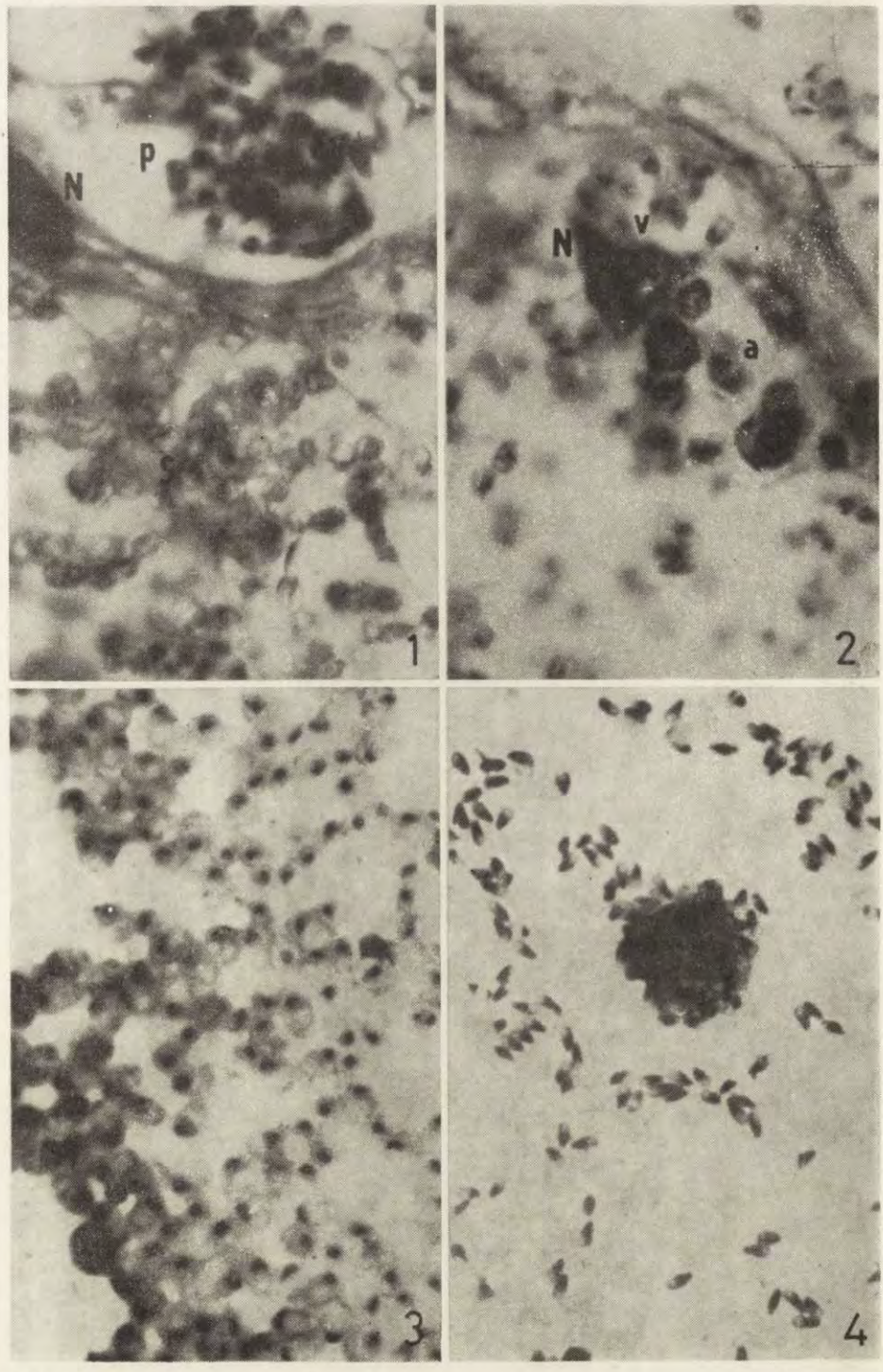

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Light and Electron Microscope Study of *Amblyospora* (*Thelohania*) *californica* (Kellen et Lipa) (*Microsporidia*) in Larvae of *Culex tarsalis* Coq. (*Culicidae*)

Received on 18 October 1980

Synopsis. The microsporidian *Amblyospora* (*Thelohania*) *californica* heavily infects the fat body cells and enocytes causing white spots or complete whitish coloration of the larvae of *Culex tarsalis* Coq. The infected cells as well as the body of the larvae undergo hypertrophy. The ultrastructure of developmental stages of *A. californica* was studied in infected host larvae. Nuclei of schizonts were frequently in diplokaryon stage. The spore has a thick exospore and a large polaroplast. The polar filament consists of 12–14 coils, 5–6 formed by the broad basal portion and 7–8 formed by the narrow distal portion.

Kellen and Lipa (1960) described from a mosquito *Culex tarsalis* Coq. a new microsporidian *Thelohania californica* which Hazard and Oldacre (1975) placed in a new genus *Amblyospora*. At the time of description only schizogony and sporogony of *A. californica* was described based on light microscope study. Later, Kudo and Daniels (1963) conducted an electron microscope study on the spore structure of *A. californica*. Kellen et al. (1965) studied the life cycle of the parasite and found the differences in its development in male and female mosquitoes.

In this paper we present results of histopathological studies on *Culex tarsalis* larvae infected by *A. californica* as well as on the ultrastructure of the life cycle of the parasite.

Material and Methods

For light microscope studies infected larvae were fixed in Bouin's fluid for 8 h and then routinely embedded into parafin. Larvae were cut on the microtome into sections 5–7 μm thick and stained in Heidenhains's hematoxylin or in Giemsa 0.25% stain for 12 h.

For electron microscope studies the infected larvae were fixed in 2% osmium tetroxide buffered at pH 7.4, dehydrated in a graded series of alcohols and embedded in methacrylate. Sections were cut with LKB ultramicrotome at 25–100 nm, stained with acetate uranyl and examined with Tesla BS 242 E electron microscope.

Results

Gross Pathology

Larvae and pupae of *Culex tarsalis* parasitized with *A. californica* have a characteristic white spots on their body. This is the result of great number of spores located in fat body cells and in enocytes and seen through the cuticule. Larvae that are moderately infected have single or a few spots. However, at the advanced stage of infection the number of spots and their size increases. At the final stage of infection practically the whole fat body is attacked by the parasite and the larva is white opaque (Pl. I 1).

Spots are most frequently observed in thorax; less frequently in abdomen and in head. This is due to the fact that fat body has a largest volume in thorax.

The volume of spores of *A. californica* is such great that the infected body parts or the whole body of the larvae is greatly hyperthrophied (Pl. I 1). The presence of a large amount of spores and other developmental stages in the tissues of the larvae influence the behaviour of the larvae. The body of infected larvae is much heavier than of healthy ones. Since the greatest number of spores is located in the thorax, this part of the larval body is the heaviest. Therefore, infected larvae frequently have a vertical position as compared with horizontal position of healthy larvae (Pl. I 1).

Light Microscopy

Fat Body

In male larvae and pupae of *C. tarsalis*, fat body were found infected. On cross sections through the bodies of infected larvae it was seen that other tissues, except the fat body, were free from the parasite (Pl. I 2). In most cases the larval fat body was so heavily infected that practically only small parts of healthy fat body were left. The gut, Malpighian tubules and muscles were not infected (Pl. I 2, 3).

The presence of huge number of spores and hypertrophied fat body caused the pressure on various internal organs of the larvae and their gut was frequently displaced from its normal position.

Enocytes

In female larvae the characteristic changes were observed in infected enocytes (Pl. II 4). They were greatly hypertrophied as their cytoplasm was completely filled with schizonts, sporonts and spores; the uninfected cell nuclei were hardly seen in cytoplasm among developmental stages of *A. californica*.

Electron Microscopy

Fat Body

The ultrastructure of infected cells showed significant changes having the character of lysis. In a weakly infected lobes and cells of fat body such structures like endoplasmic reticulum, mitochondrions, ribosomes and other showed only minor pathological changes. However, as schizonts and sporonts grew and increased their number, they disrupted the cell membranes causing that the fat body lost its cellular structure (Pl. II 5). In a fluid resulting from a destruction of large lobes of fat body mitochondrions were well seen which preserved their structure quite long.

Schizonts, Sporonts and Sporoblasts

Schizonts observed on ultrathin sections were either uninucleate or binucleate; tetranuclear schizonts were not observed. The nuclei of schizonts in diplokoryon stage are paired with their flattened sides in contact (Pl. II 5). The nuclear membrane along the plane of apposition is thicker than in other regions. The nuclei of diplokaryons and of uninucleate forms are large and occupy relatively large volume of schizonts. The nucleoplasm consists of homogenous material of about the same density as the cytoplasm (Pl. II 6, 7).

Dividing of sporonts is by binary fission and various phases of their separation could be observed (Pl. III 8).

During sporont and later sporoblast differentiation the plasma membrane became more distinct, while the cytoplasm became more vacuolized (Pl. II 7, III 8).

The metabolic granules formed at the early phases of development did not disappear and were well seen inside the pansporoblast at the further stages of spore maturation (Pl. II 7, III 8, 9).

Spore

In young and mature spores all structures typical for a microsporidian spore were readily seen (Pl. III 9, 10). The nucleus was relatively large as well as the polaroplast. The sporoplasm in young spores was more compact and electron dense (Pl. III 9).

Beginning of filament formation was seen in an early sporoblast. On sections of mature spore around the central core of the polar filament an outer wall is well seen (Pl. III 10).

The polaroplast occupies one third of the spore volume. It is mostly formed by small vesicles (Pl. III 9, 10).

The polaroplast is closely associated with the manubrial part of polar filament (Pl. III 10). The number of polar filament coils equals 12–14.

Near the posterior pole of the spore the posterior vacuole was seen (Pl. III 10).

Most spores seen on ultrathin section is oval or ellipsoidal. However, some spores had a truncate or invaginated appearance (Pl. III 10) evidently due to fixation and dehydration procedure.

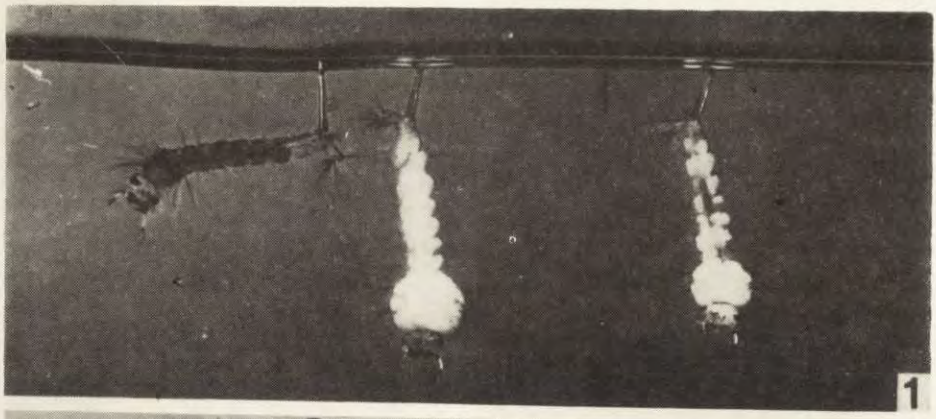
Discussion

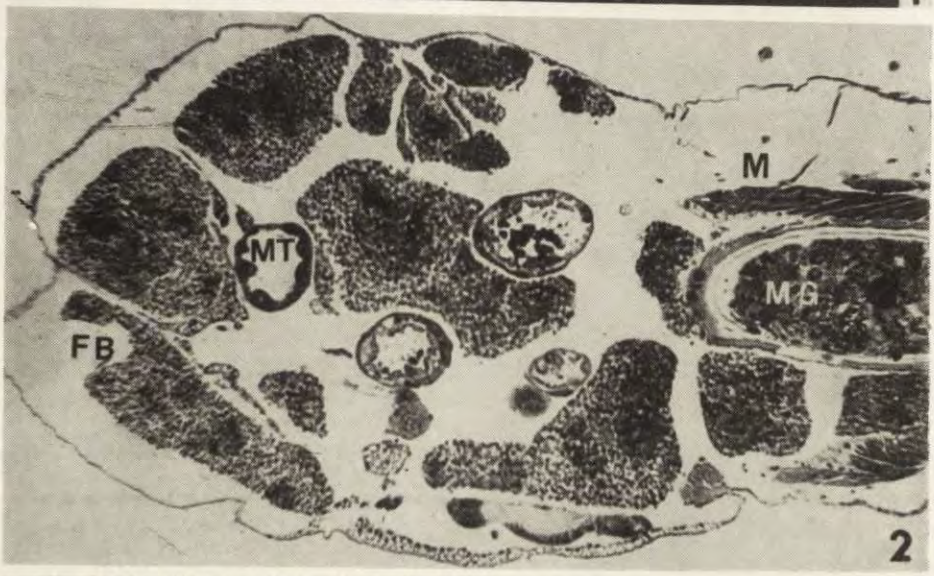
The light and electron microscope study of tissues of *C. tarsalis* larvae showed the great histopathological changes in fat body cells and enocytes caused by *A. californica*. These changes explain the great extension of the development time of larvae and their high mortality reported by Kellen and Lipa (1960).

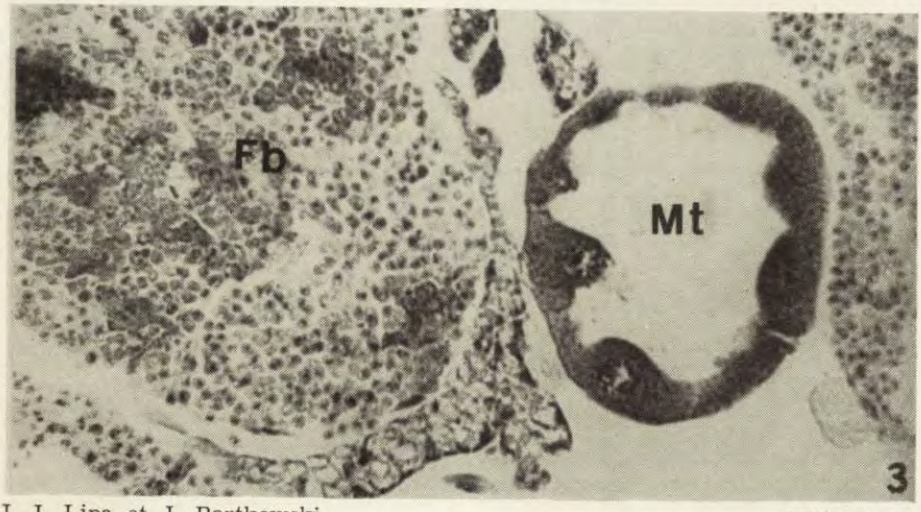
Our observations on ultrastructure of spore of *A. californica* are in agreement with results of studies by Kudo and Daniels (1963). The nucleus of the spore is elongated and occupies great part of sporoplasm. The polaroplast is laminated in its upper part and reticulated in lower (Pl. III 9, 10). The number of polar filament coils observed by us was 12–14 (Pl. III 10). The polar filament was occasionally doubly coiled (Pl. III 9, 10). Kudo and Daniels (1963) also observed in most spores 12–14 coils but in some spores 11 to 16 coils were seen.

The metabolic granules observed by us inside pansporoblastic membranes (Pl. II 5, III 7, 8) were formerly reported by Kudo (1924) and Hazard and Oldacre (1975) but their role remains unknown. According to the latter authors the granules are secreted by dividing sporonts.

The general feature of spores of many *Amblyospora* species is that after fixation they are truncate what is well seen in light microscope. This feature is well seen also on electron micrographs (Pl. III 10) and evidently can be explained by the fact that exospore is thinner in the apical end but frequently at the posterior too. Therefore some spores are truncate at both ends. The thinner exospore at the apical end secu-







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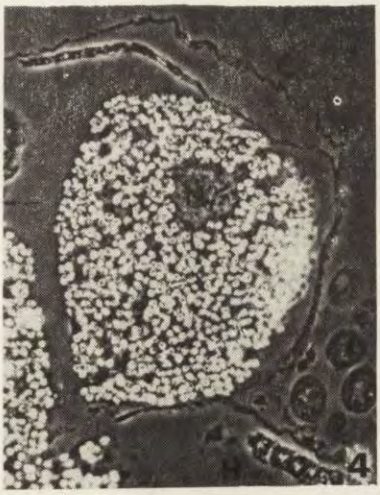

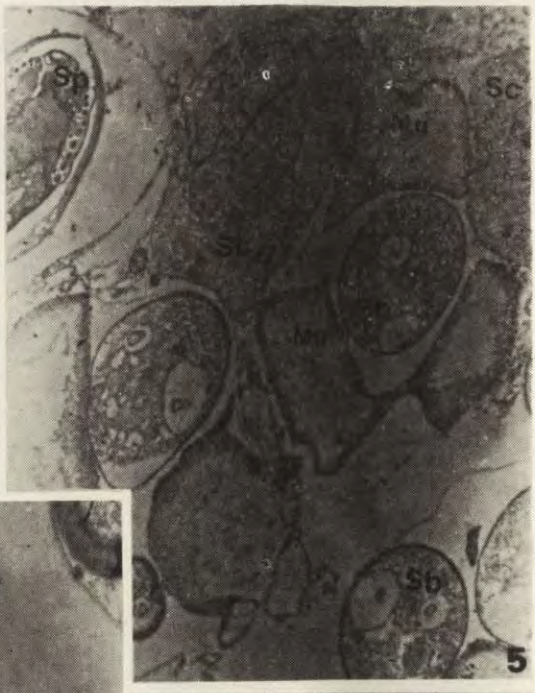

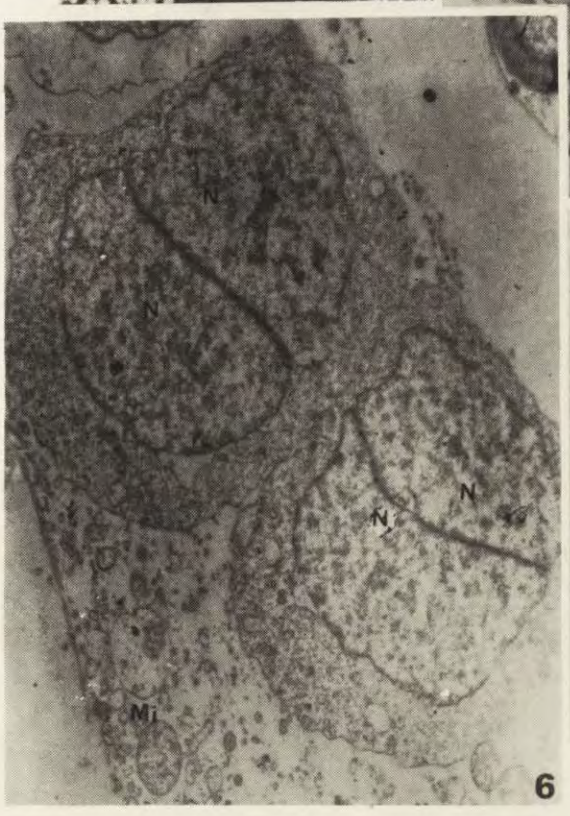

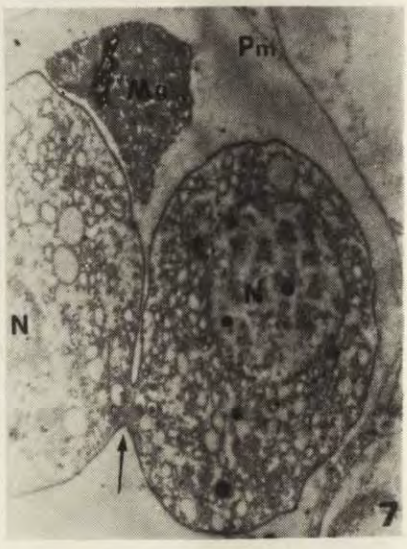

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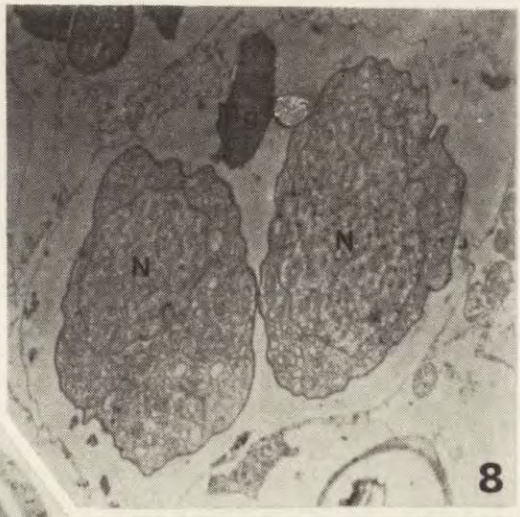

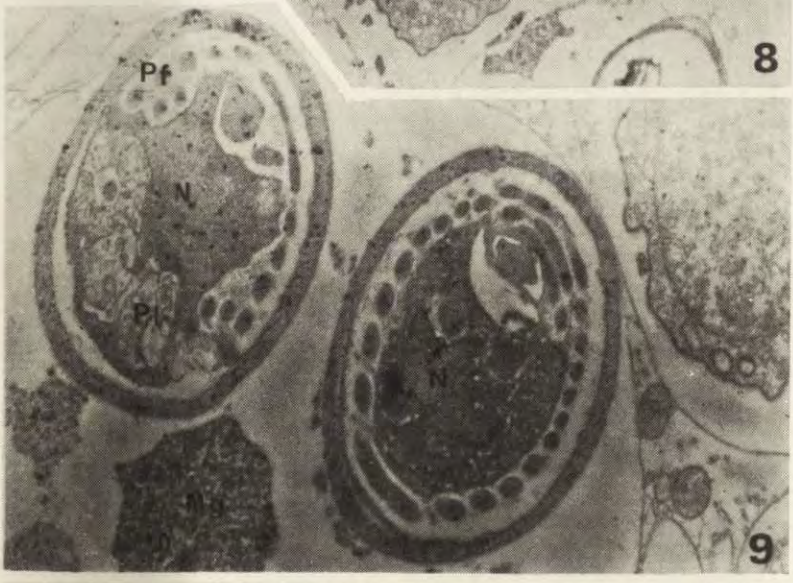

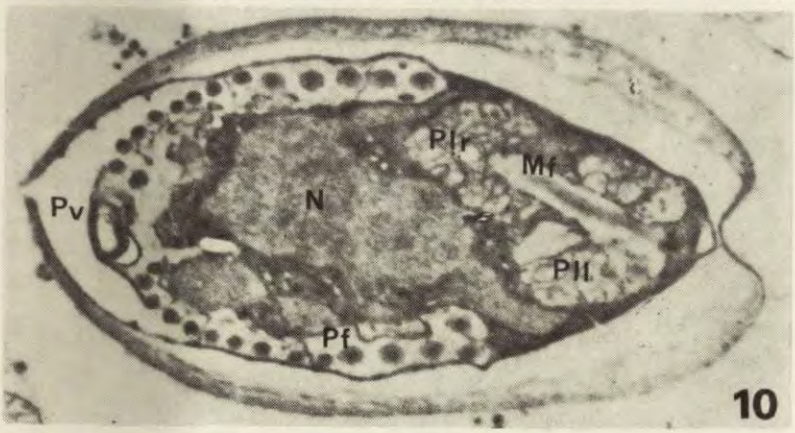

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res the appriopriate work of polaroplast and quick polar filament extrusion.

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EXPLANATION OF PLATES I–III

1: Healthy (left) larva of *Culex tarsalis* Coq. and heavily infected (right) by *Amblyospora californica*

2: A section through a body of larva with fat body (FB) heavily attacked by *A. californica*; malpighian tubules (MT), muscules (M) and midgut MG) are free from infection

3: A huge number of spores and other developmental stages of *A. californica* in larval fat body (Fb); muscules (M) and malpighian tubules (Mt) are not infected

4: Cytoplasm of larval enocyte filled with spores: N-nucleus of enocyte, C — cuticule, H — hypodermis

5: Electron micrograph of fat body heavily infected with schizonts (Sc), sporoblasts (Sb) and spores (Sp) of *A. californica*; metabolic granules (Mg) seen inside pansporoblastic membrane (8400 ×)

6: Electron micrograph of two schizonts in diplokaryon stage: N-nucleus of schizonts, Mi — mitochondrion of fat body cell (8400 ×)

7: Two sporonts in the final stage of division: N — nucleus, MG — metabolic granules (8400 ×)

8: Electron micrograph of two sporonts within pansporoblastic membrane (Pm): N — nucleus, Mg — metabolic granule (4000 ×)

9: Two young spores at the final phase of maturation: N — nucleus, Pf — polar filament, Pl — polaroplast, Mg — metabolic granule (10 000 ×)

10: Mature spore: N — nucleus, Plt — laminated polaroplast, Plr — reticulated polaroplast, Mf — manubrial part of filament, Pf — polar filament coils, Pv — posterior vacuole (25 000 ×)

NOTICE TO AUTHORS

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

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

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

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The following material should be supplied on separate sheets 1. the running title for the page headlines, 2. tables, 3. legends for text figures, 4. legends for plates. Line-drawings will be published in the text, photographs and raster figures on separate plates. No coloured photographs can be published presently. Lettering on photographs and drawings should be marked in pencil. With no regard to the language of the text, only the Latin lettering, arabic numerals or generally accepted symbols are admittable for marking on illustrations. Numbering of text-figures, plates and tables must be also marked in pencil, as well in the legends as in the text.

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In Preparation:

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