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Conjugation-specific Cortical Reorganization During Zygocyst Formation in the Hypotrich Ciliate *Paraurostyla weissei*

Małgorzata FRONTCZAK-BANIEWICZ and Maria JERKA-DZIADOSZ

Department of Cell Biology, M. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Summary. The changes of ciliary structures during total isogamontic conjugation of *Paraurostyla weissei* were investigated with the aid of scanning electron microscopy (SEM). Total conjugation involves absorption of the left mate (donor) by the right mate (recipient) with concomitant resorption of preconjugant ciliature: first in the left, then in the right mate (Jerka-Dziadosz and Janus 1975). Three rounds of cortical reorganizations take place during sexual processes in this ciliate. The first (zygotic) reorganization, overlapping with absorption of donor and resorption of the prezygotic ciliature is highly modified. The zygotic-specific primordial pattern of dorsal streaks gives rise to a transient corticotype confined to the zygocyst stage. The "dorsal" units spread over most parts of the zygocyst surface and undergo a deciliation-reciliation cycle.

The second reorganization occurs in the exconjugant zygocyst. Basal body proliferation of oral, ventral and dorsal primordia is initiated close to the "dorsal" basal bodies scattered throughout the zygocyst yielding complete set of primordial structures differentiating into smaller number of ciliary structures which is next replaced in the third cortical reorganization restoring normal ciliary pattern.

We suggest that the alternative cortical organization of the zygocyst is related to that of conjugation-specific intracytoplasmic cytoskeleton functioning in segregation of the products of nuclear differentiation.

Key words. Conjugation, ciliary structures, reorganization, ciliates, *Paraurostyla*.

INTRODUCTION

During conjugation the hypotrich ciliates commonly reorganize their cortex more than once: twice in *Euplotes*, thrice in *Stylonychia* and *Paraurostyla* (Review in Ng 1990).

In *Paraurostyla weissei* the conjugation is total - that is the left conjugant (the donor) becomes absorbed into its mate (the recipient) (Heckmann 1965, Heumann 1975). Previous account of cortical development based on Protargol staining (Jerka-Dziadosz and Janus 1975)

revealed that the first reorganization which begins soon after pairing is highly modified: (1) the proliferation of basal bodies occurs only in the recipient, (2) no ventral primordia are formed, except much reduced oral field, quickly resorbed, (3) the dorsal bristle set is complete except for caudal cirri normally differentiating at posterior termini of some dorsal rows, (4) the proliferation of basal bodies and differentiation of the incomplete ciliature occurs concomitantly with resorption of the pre-conjugant ciliature, which starts soon after pairing with resorption of the oral ciliature.

After fusion of mates the remnants of preconjugant ciliature disappear and a zygocyst is formed. The first cortical reorganization proceeds during pronuclear

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Address for correspondence: M. Frontczak-Baniewicz, Department of Cell Biology, M. Nencki Institute of Experimental Biology, Polish Academy of Science, 3 Pasteur Str, 02-093 Warsaw, Poland.

migration, fertilization and division of synkaryon. It is therefore termed the "zygotic reorganization".

The second reorganization occurs in an early exconjugant zygocyst, soon after the new macronucleus has differentiated and although all categories of ciliature are formed (one set) the corticotype is incomplete and normality is restored by the third reorganization, which resembles a physiological reorganization (Jerka-Dziadosz and Frankel 1969).

This study was aimed at elucidation of the fate of the dorsal primordia originating in the first reorganization and their role in continuation of the ciliary pattern. We found that the morphogenetic pattern of zygotic development of dorsal units is unique and differs from asexual reorganization; it gives rise to a transient corticotype confined to the zygocyst stage at which the dorsal units are not morphogenetically quiescent. We suggest that this alternative cortical organization is related to conjugation-specific differentiation of the intracytoplasmic cytoskeleton necessary for normal segregation of the products of nuclear differentiation occurring in the zygocyst.

MATERIAL AND METHODS

Cells and cultures

Cells used here were the stocks of *Paraurostyla weissei* isolated from natural sources in Poland and France, and progeny lines obtained after crosses in the laboratory. The following cell lines were used: LuV, N1Sav and line 9. Stocks Vernier (V) and Savage (Sav) were kindly supplied by Dr. A. Fleury. The origin of lines: Lu, N1 and 9 was described previously (Jerka-Dziadosz et al. 1989, Jerka-Dziadosz and Wiernicka 1992). The cells were cultivated in Petri dishes in a modified Pringsheim solution (Jerka-Dziadosz and Frankel 1969) and fed with flagellate *Chlorogonium sp.* grown on soil extract (Heckmann and Kuhlmann 1986).

Induction of mating

Cells representing complementary mating types were mixed. *Tetrahymena thermophila* washed from 2% proteosopeptone was added to cause massive conjugation. Mating started 5 to 24 hours after mixing.

Light and scanning electron microscopy

For routine observations of changes on the cell surface, the silver proteinate (Protargol, Merck) staining was used as described previously (Jerka-Dziadosz 1985). Some samples were stained with lacto-orceine (Miyake et al. 1979) for observations of changes in the nuclear apparatus. For scanning electron microscope, cells were fixed in 1:1 (vol) mixture of 4% osmium tetroxide and saturated mercuric chloride solution for 30 min, washed in distilled water and
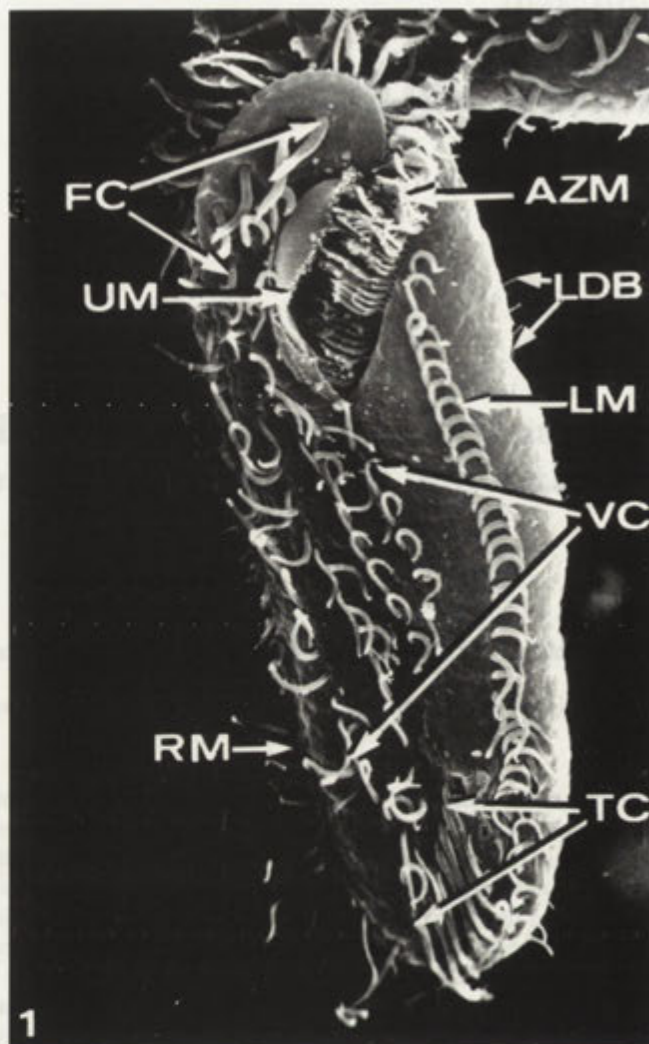

Fig. 1. Ciliary structures on the ventral surface of *Paraurostyla weissei*. The oral ciliature consists of: adoral zone of membranelles (AZM) and undulating membrane (UM). The cirri consist of four groups: frontal cirri (FC), ventral cirri (VC), transverse cirri (TC) and single rows of marginal cirri located at the left (LM) and right (RM) margins. The dorsal surface is covered by rows of dorsal bristles. The left-most dorsal row (LDB) is visible. x700

dehydrated with graded series (50-100 percent) of ethyl alcohol followed by amyl acetate. Next, cells were critical - point dried, then coated with carbon and gold particles before observations in JEM 1000 electron microscope.

RESULTS

The organization of ventral surface of interphase *Paraurostyla weissei* is shown on Fig. 1 and described in the legends. When two complementary mating types are mixed tight pairs are formed and subsequently the left partner (donor) is absorbed by the right one (recipient). The ciliature of both partners is gradually resorbed, first in the donor, then in the recipient (Figs. 2, 3) and is
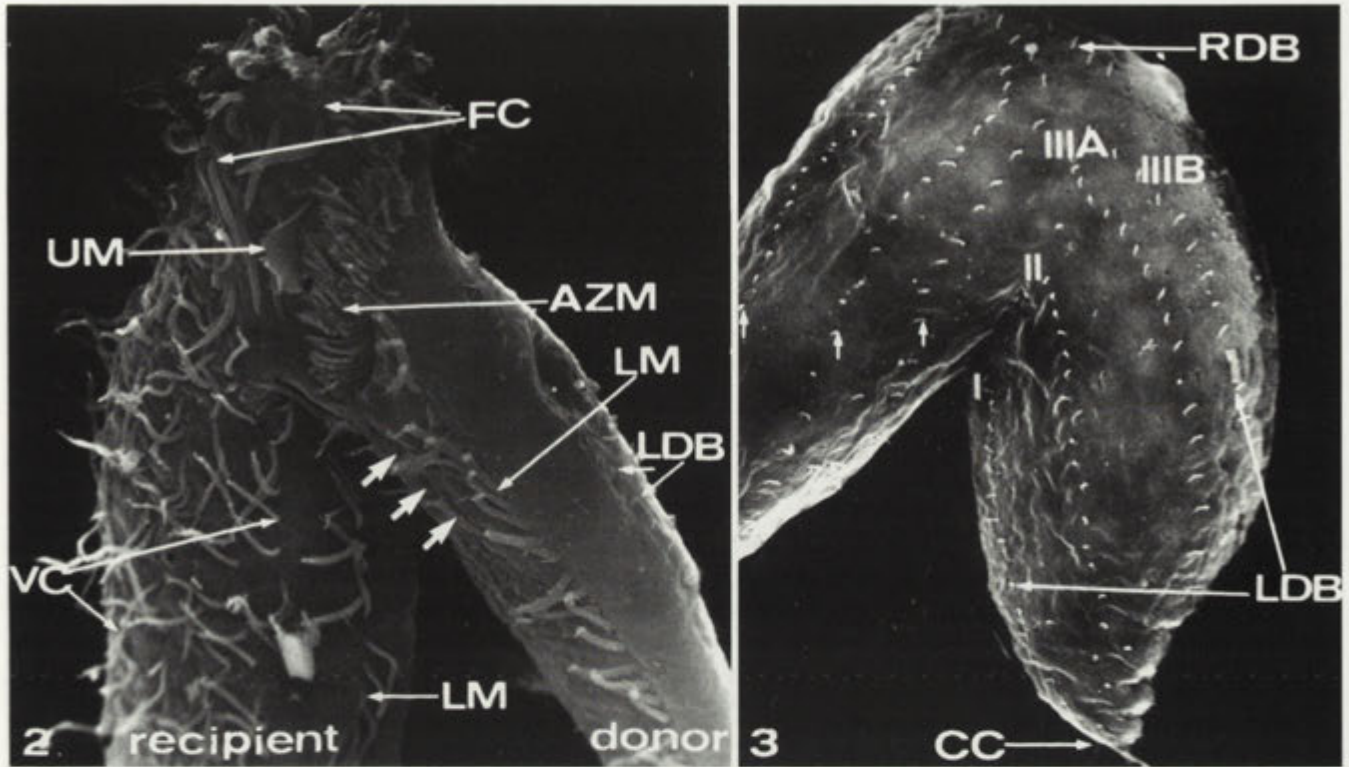
Fig. 2. The ventral surfaces of the conjugating pair. One common AZM is seen. On the donor the frontal cirri have been resorbed, the ventral cirri (arrows) are resorbed. The left marginal cirri and dorsal bristles are present. In the recipient only part of oral structures has been resorbed. x850

Fig. 3. Dorsal surface of a pair: four rows (I-IIIB) of the left dorsal bristle rows and two right bristle rows (RDB) are visible in the recipient. Arrows point to the dorsal rows in the donor. CC-caudal cirri. x400

replaced by one incomplete set developing in the recipient during the first cortical reorganization (Jerka-Dziadosz and Janus 1975).

SEM studies allowed to follow the fate of the much reduced ventral primordia and development of the primordia of the dorsal bristles. On the ventral surface (Figs. 4a, 4c) a small primordium is formed between the ventral and the left marginal cirral row. The basal bodies in this primordium do not bear the kinetodesmal fiber (unpublished immunocytochemical studies), thus they correspond to oral category (Jerka-Dziadosz 1990). They do not differentiate into definite membranelles, rather they regress before the remaining of the old ventral ciliature is resorbed (compare Fig. 7). Occasionally single or paired 3-4 very short cilia persist in the mid-ventral region of the zygocyst.

A primordial group of cilia is formed within the old right marginal cirral row (Figs. 4a, 4b). These primordia arise with participation of the old marginal cirri (compare Fig. 10 in Jerka-Dziadosz and Janus 1975). They differentiate into pairs with one basal body ciliated, the other one barren (Jerka-Dziadosz 1982), similarly as the right dorsal bristle units differentiate in physiological

reorganization, except that during conjugation primordia of the right marginal cirri are not formed (Fig. 7). Next, these primordia differentiate into right dorsal rows. The new units move onto the dorsal side of the recipient, they disperse rather than forming ordered rows of bristles.

On the left dorsal side of the recipient three longitudinal streaks of short paired cilia are formed within old rows (Fig. 5). In the first left dorsal row at the beginning we observed one uninterrupted row of short paired cilia (about 48-50 pairs). The anterior cilium of each pair grows further and the posterior one shortens as in normal morphogenesis (Jerka-Dziadosz 1982). In the next stage the primordium breaks into segments and most frequently forms about five short oblique rows of bristle units (Figs. 6,7). The posterior ends of the segments are directed toward the ventral side (Fig. 7). The new dorsal primordia within the second and third row of bristles (Figs. 5,6) develop as in physiological reorganization. The third streak differentiates into two overlapping segments. Caudal cirri differentiating in physiological reorganization from the posterior pairs of dorsal primordia are not formed during zygotic reor-
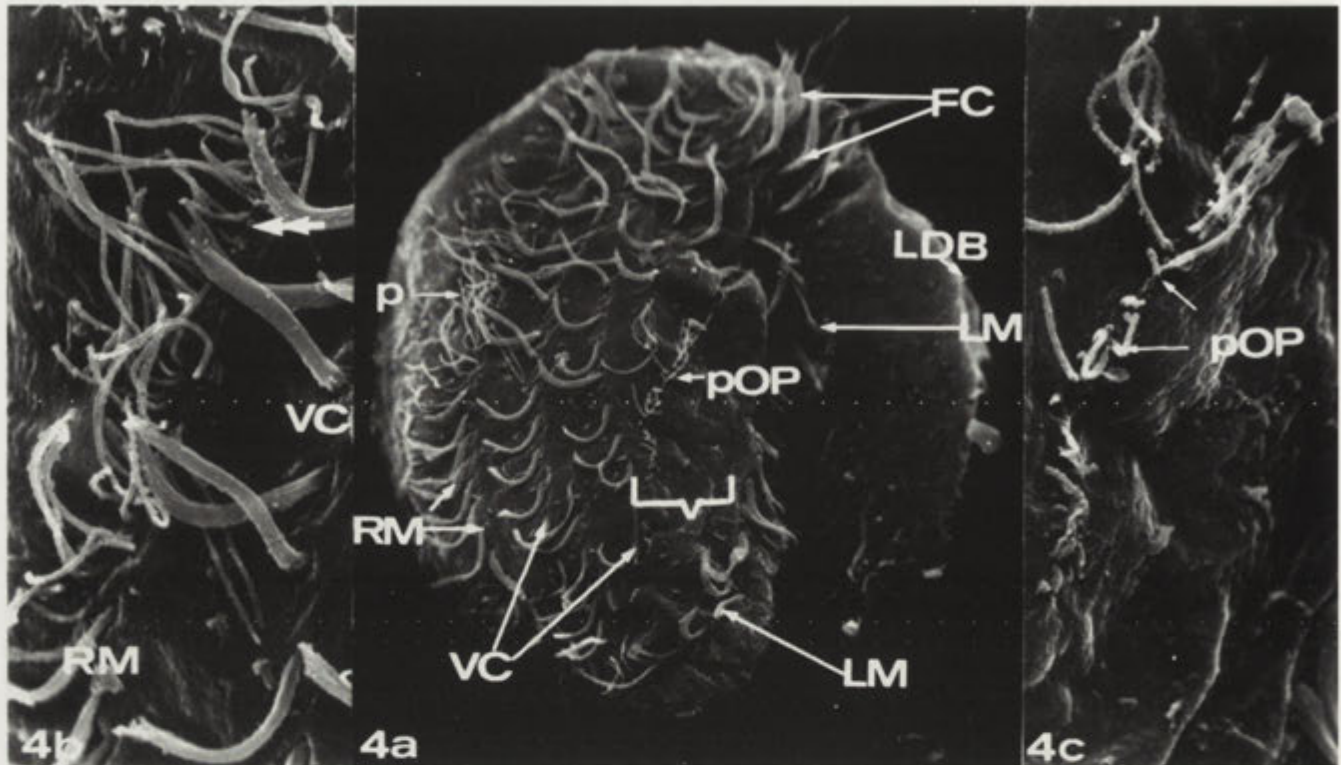
Figs. 4a-c. 4a-Ventral surfaces of the conjugating pair shortly after fertilization. In the donor remnants of LM and LDB are visible. In the recipient transverse and posterior ventral cirri are absent. Primordial group of cilia (p) within the old RM and rudimentary oral primordium (pOP) between the ventral and the left marginal cirral row (bracket) are visible. x240. 4b-Magnification of the RM with developing primordium. The cilia from old cirri are dispersed. Double arrow point to a short cilium of a ciliary pair. x5000. 4c-Magnification of pOP. Dispersed cilia and short stubs are visible. The primordium regresses. x5800

ganization. The new bristle units spread sideways and polarly and shortly lose the paired aspect. Only one club-shaped short cilium remains (Fig. 8a, 8b) in each unit.

Formation of new ciliary structures on the dorsal surface occurs simultaneously with resorption of the old ciliature on the ventral surface and absorption of the donor. The old dorsal bristles are still present after complete resorption of oral and ventral ciliary structures. Later on they shorten and are no longer distinguishable from the newly formed bristles. Most probably at least part of them is eventually resorbed.

After absorption of the donor the zygocyst takes the shape of an irregular sphere. The greatly reduced ventral surface is seen as a depression in the sphere. The old ventral and marginal cirri are resorbed within this depression. Afterwards, longitudinal protrusions and folds are visible on the zygocyst (Fig. 8). Single, very short dorsal cilia are dispersed randomly on almost the whole surface of the zygocyst but there are less of them on the ventral part. That stage of ruffled sphere lasts about 100 hours, no visible changes were noticed on the surface in SEM images.

Before the second cortical reorganization transiently two populations of ciliary units were distinguishable (Fig. 9): there are units consisting of ciliary pairs and single cilia. It seems that cilia may regrow on basal bodies which persisted under the membrane throughout

Fig. 5. Ciliary primordia on the dorsal surface of the recipient. The short paired cilia are formed within the old dorsal rows I-III. The first left dorsal streak contains overlapping segments (arrows). Note that cilia in a pair are of unequal length. The old bristles are visible (arrowheads). x6300

Fig. 6. A conjugating pair seen from the left side. In the pointed, posterior part of the donor (star) bristles are absent, arrowheads point to resorbing bristles. In the recipient the first left dorsal primordium (I) contains several (arrows) oblique rows of cilia. Arrowheads point to old bristles. x1800

Fig. 7. Recipient seen from the anterior side. The ventral part of AZM and some frontal cirri are absent. New right (pRDB) and left (pLDB) dorsal bristles are visible. Note: short oblique rows of pLDB. In the left-ventral region of the cell (bracket) where the rudimentary pOP was formed during the first cortical reorganization (compare Fig. 4a), no cilia are visible. x1600

Fig. 8a-8b. 8a-The zygocyst with randomly dispersed single club-shaped short cilia (arrows). Longitudinal protrusions and folds are visible on the zygocyst. x1200. 8b-Higher magnification of the club-shaped short cilia. x11000

the zygocyst stage. Transmission electron microscopy and immunocytochemical studies will clarify this matter. The mixed-population stage lasts a short time and shortly before the formation of the new primordia on the surface of the zygocyst there are only single cilia, their length corresponding to normal cilia (compare Fig. 10 and 13).

From previous studies it is known that one set of a new ciliature develops in an exconjugant. In SEM images at first the oral primordium is visible as a longitudinal field of short cilia located in a mid-ventral depression of a zygocyst. It seems that proliferation of basal bodies is initiated around pre-

existing ciliated basal bodies (Fig. 10) as some of the short bristles can be identified within an anarchic field. Still in the depression longitudinal streaks of the UM and FVT primordia develop. Outside the depression on both margins of the ventral surface the marginal streaks of paired cilia appear (Figs. 11, 12).
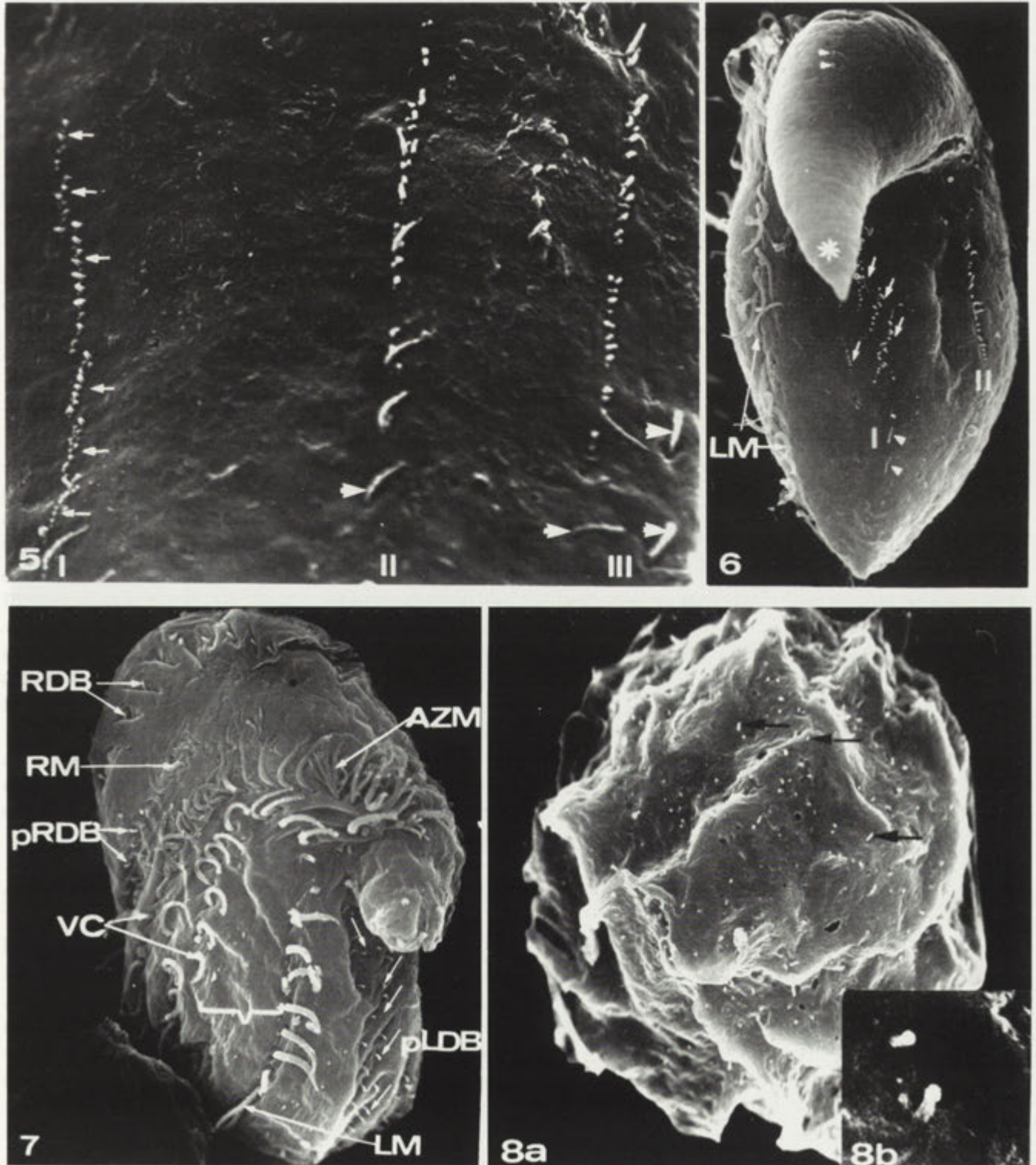
On the dorsal surface of the exconjugant formation of pairs of new short cilia in three longitudinal rows occurs. As on the ventral surface proliferation of basal bodies is initiated at specific locations close to some of the bristles present in the zygocyst. The third dorsal primordium splits into two overlapping streaks and forms the new rows IIIA and IIIB. Caudal cirri are formed at posterior termini of rows I, II and IIIB (Fig. 13) as in physiological reorganization (Jerka-Dziadosz and Frankel 1969). After this reorganization in the exconjugants all categories of ciliary structures appear but the number of differentiated structures is smaller than in normal cells. Usually only 3-5 frontal cirri (instead of normal 7) and 3-5 transverse (instead of normal 7-8) cirri differentiate (Jerka-Dziadosz and Janus 1975). The remnants of the zygotic bristles are resorbed during differentiation of the exconjugant ciliature.

The third cortical reorganization involves replacement of all cortical structures formed during the previous reorganization by a complete set of ciliary structures (Fig. 14). That process is similar to normal physiological reorganization. The remnants of the exconjugant ciliature are decomposed after the new set differentiate.

DISCUSSION

The main conclusions emerging from this study are the following:

1. The first cortical reorganization (zygotic) although highly abbreviated on the ventral side (no cirri or membranelles are formed), provides abundant dorsal units which spread all over the surface of the zygocyst.

2. The morphogenetic pattern of dorsal unit differentiation is highly modified, especially in the first left dorsal row. Caudal cirri are not formed.

3. The dorsal units persist throughout the zygocyst stage with the basal bodies going through a ciliary shortening-regrowth cycle.

4. Some of the zygotic dorsal units become incorporated not only into dorsal, but also into oral and ventral primordia during the second reorganization taking place in the exconjugant.

In previous accounts of total conjugation in *Paraurostyla weissei* (Jerka-Dziadosz and Janus 1975, Jerka-Dziadosz and Dubielecka 1985) based on Protargol stained cells it was suggested that the ventral ciliature of the exconjugant appears on a completely "naked" cortex lacking any pre-existing ciliature, therefore the primordia apparently would have formed de novo. Present study based on SEM and TEM (Frontczak unpublished observations) supported by immunocytochemical studies (Fleury et al 1988, 1991) allow to qualify this conclusion.

Ultrastructural studies of the surface cytoskeleton of vegetative cells (Jerka-Dziadosz 1982) and zygocysts (Jerka-Dziadosz 1984) revealed that the superficial microtubular cytoskeleton is retained throughout the whole zygocyst stage. Immunocytochemical studies additionally revealed that this cytoskeleton (Fleury and Laurent 1991) and the dorsal ciliary units undergo specific transformations (Frontczak and Jerka-Dziadosz 1990) resulting in continuation of cellular polarity and asymmetry, through formation of transient corticotype consisting only of single or paired basal body units.

The transient corticotype originates from a conjugation-specific morphogenetic pattern which does not resemble the one of the incipient opisthe in binary fission nor the one of normal physiological reorganization. The lack of ventral structures and specific modifications in differentiation of the first left dorsal row (Fig. 6) support the interpretation that the zygotic reorganization is unique and specific for this stage of the life cycle in *Paraurostyla*. It provides cytoskeletal nucleating centers for the organization of the zygocyst subsurface and intracellular cytoskeleton necessary for normal segregation of the products of nuclear differentiation, and seeds for reconstructing of single cortical pattern, in the much postponed "sexual" reorganization taking place in the exconjugant.

The morphogenetic patterns of the zygotic reorganization in hypotrichs have been interpreted by Ng (1990) as resulting from heterochrony and overlap of asexual and sexual processes. Ng (1990, page 83) noted that: "control of ciliate morphogenesis in sexual reproduction differs from that in asexual reproduction, even though the morphogenetic processes in the two situations are similar". Our present study conform to the first part of that statement but disagree with the second one. Morphogenetic processes occuring during the first cortical reorganization ("zygotic") and the second one ("sexual") as well as the morphogenetic activity taking
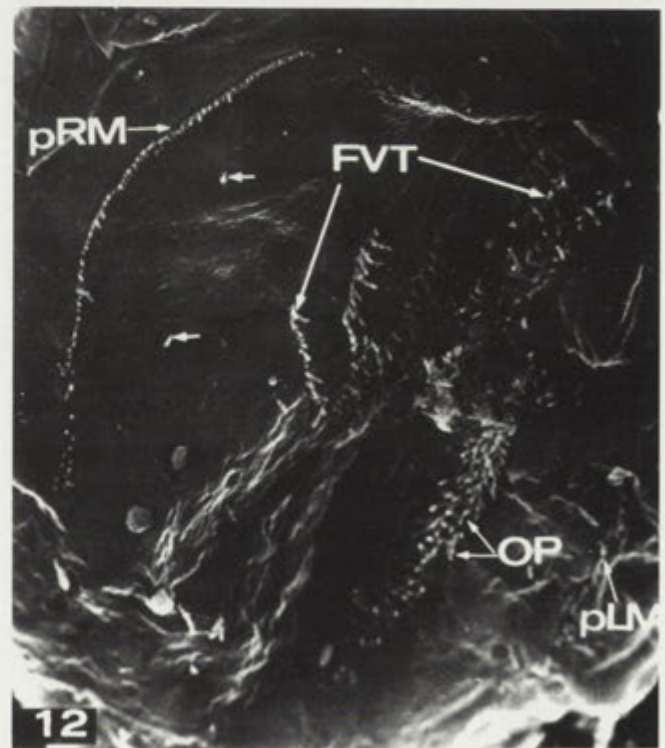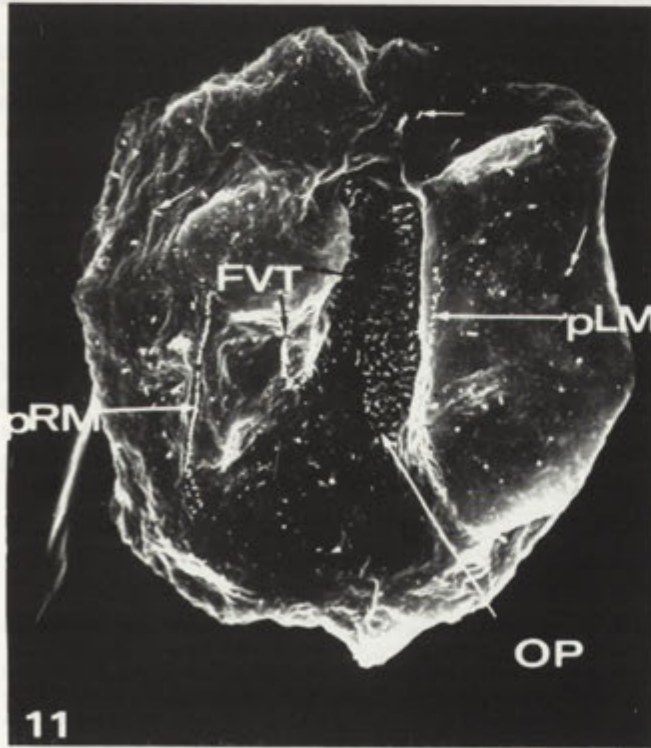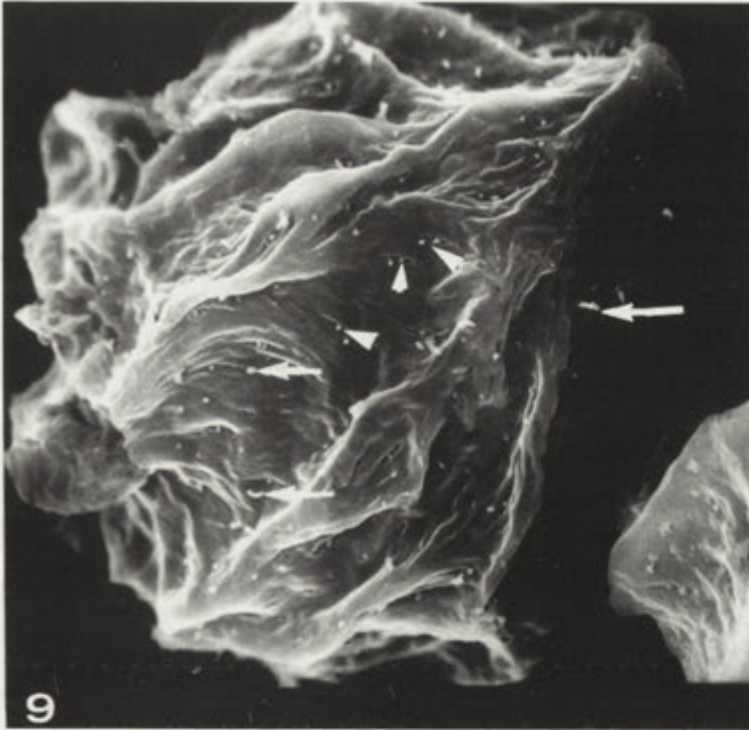
Fig. 9. The late zygocyst before the second cortical reorganization. Two populations of ciliary units containing: single longer bristles (arrows) and paired short stubs (arrowheads) are visible. Note the wrinkled surface of the zygocyst. x2500

Fig. 10. An exconjugant at the early stage of formation of oral primordium. The ventral surface is evaginated due to the fixation. The oral primordium is visible as groups of short cilia forming a longitudinal field. Arrows point to preexisting short cilia located within and close to the primordium. x11000

Fig. 11. The primordia of ventral ciliature in the early exconjugant. The oral primordium (OP) and fronto-ventral-transverse cirri (FVT -black arrows) develop in the depression. At the edges of the depression primordial streaks of marginal cirri (pLM and pRM) are present. Arrows point to preexisting bristles. x1100

Fig. 12. The ventral primordia in the exconjugant. Note the old short bristles on the ventral surface (arrows). x4500

Fig. 13. The primordia of dorsal ciliature in the exconjugant (I-IIIB). New CC at posterior end of the row IIIB. Arrows point to old bristles. Note to almost equal length of old and new bristles. x1050

Fig. 14. The ventral side of an exconjugant fixed during the third cortical reorganization. Complete FVT replace the first generation of exconjugant cirri. Note four old TC and seven new TC (compare with Fig.1). x850
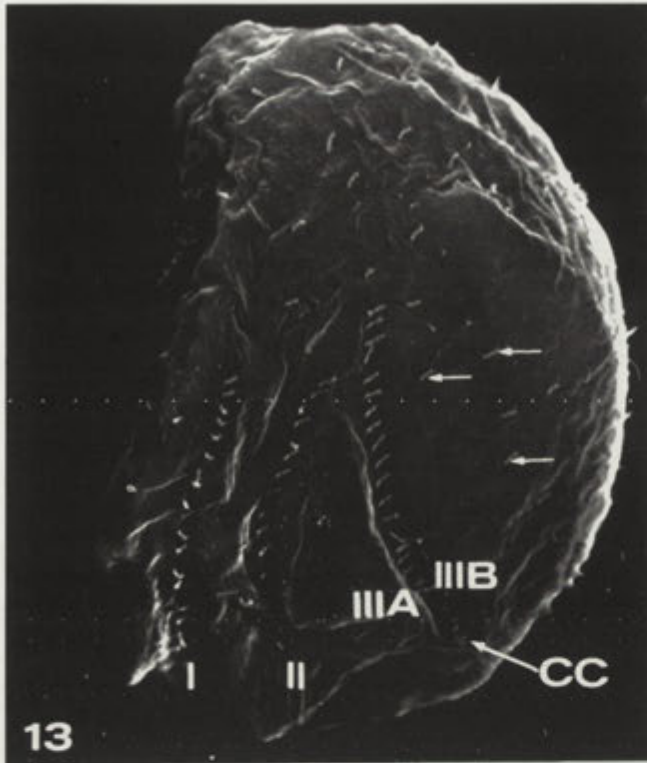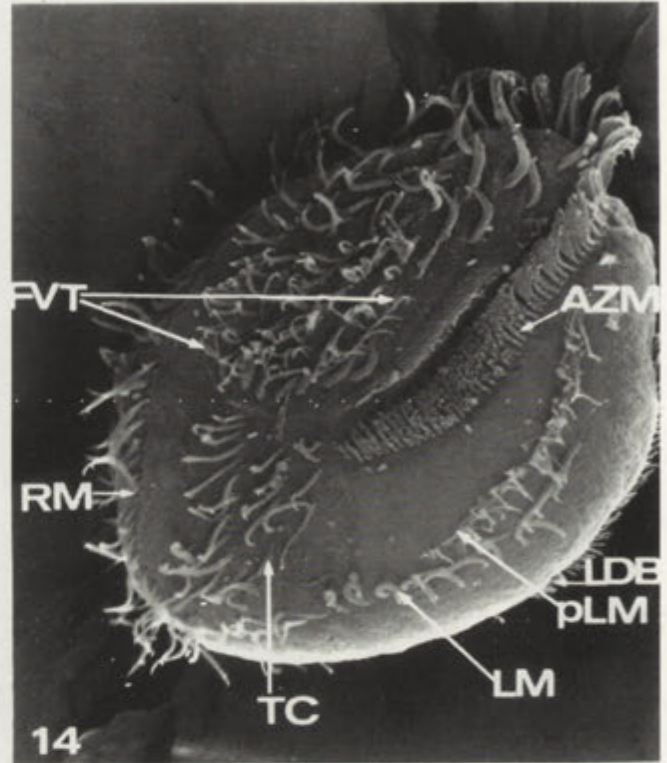
place in between these two events in *Paraurostyla weissei* do differ significantly from normal asexual development (Jerka-Dziadosz and Frankel 1969, Jerka-Dziadosz and Janus 1975). This difference may partly be explained by an overlap of divisional and encystment resembling processes with that of sexual processes, all of which affect the cytoskeleton in the first cortical reorganization, and the reduced corticotype originating in the second reorganization (Fig. 14) could be related to much reduced size of the exconjugant (Jerka-Dziadosz 1976). The intracellular aspect of the cytoskeletal dynamics and its coexistence with the sub-surface systems (compare Cole 1991), much neglected in studies on conjugation of hypotrich ciliates, could provide a clue for clarification of the problem of "embryological" morphogenesis in these ciliates.

An interesting observation noticed in this study is the dynamics of the dorsal units in the zygocyst. Although SEM images provide only limited information concerning these structures the transition from double basal body units to single ones as well as modification in the ciliation status indicate that they are not quiescent throughout the zygocyst stage. Immunocytochemical studies now in progress, should provide more informa-

tion concerning the dynamic relations between the sub-surface and intracellular cytoskeletal elements taking place during nuclear differentiation.

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Macronuclear DNA and Total Protein Contents in Differently Cultured Cell Lines of *Paramecium primaurelia* Mating Types

Maria Umberta DELMONTE CORRADO[*], Massimo COSTANTINI[**] and Tina CRIPPA FRANCESCHI[*]

[*]Istituto di Zoologia dell'Università, Genova, Italia, [**]Servizio di Epidemiologia Clinica e Sperimentazioni Controllate, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italia

Summary. The macronuclear DNA and total protein contents of *Paramecium primaurelia* stock 90, were measured cytofluorometrically in differently cultured cell lines of mating type I and mating type II. One method allowed the lines of both mating types to develop their different growth rates from conjugation to reactivity (non-synchronized lines); the other method selected lines from populations of the two mating types, that were growing at similar fission rates as possible to each other (synchronized lines). The results indicate that, under reactivity conditions, mating type I cells show significantly or not significantly higher macronuclear DNA and total protein contents than mating type II cells, in case the culture method respectively did not or did synchronize the lines of the two types. Moreover, both macronuclear DNA and total protein contents of log-phase growing synchronized cells of the two mating types, vary for the interaction between fission age and mating type, in the range between 10 and about 26 fissions following conjugation. The different growth rates of mating type I and mating type II cell lines, evidenced by failure in thoroughly synchronizing them, are assumed to be consistent with their differential macronuclear DNA and total protein contents; consequently, a relative autonomy of the mating types can be stressed.

Key words. Macronuclear DNA, protein content, *Paramecium primaurelia*, mating types.

INTRODUCTION

Studies on the mating types of *Paramecium primaurelia* showed that different rates of growth characterize the mating type I and the mating type II cell lines from sexual reorganization to reactivity (Crippa Franceschi 1981, 1987); moreover, that the mating reactive populations of the two types differ from each other in their macronuclear DNA content (Delmonte Corrado 1987) and in cell density (Crippa Franceschi 1987, Ramoino 1988), as well as in a number of responses related to: autogamy occurrence time (Crippa Franceschi

et al. 1989 b), electromigration speed (Crippa Franceschi et al. 1989 a), rate of food vacuole formation (Ramoino 1989), effect of a vital dye uptake both on macronuclear DNA content (Delmonte Corrado et al. 1992) and cell size (Ramoino et al. 1992).

These differences took place when the classical method of cells culturing was used (Sonneborn 1950, non-synchronized lines), which allowed the two mating type lines to develop their different rates of growth from sexual reorganization to reactivity. The differences shown under reactivity conditions, were thought to be related to the differential growth of the populations of the two types. A molecular model referred to mating type expression (Morchio and Crippa Franceschi 1986, 1989) infers that different protein amounts would

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Address for correspondence: M. U. Delmonte Corrado, Istituto di Zoologia dell'Università, Via Balbi 5, 16126 Genova, Italia.

characterize the ciliary surface of the cells of the two types involved in the mating. A higher protein concentration is assumed to be present in mating type I, whose cell lines show slower growth and have completed a lower number of fissions; while, a lower protein concentration is thinkable in mating type II, whose cell lines show more rapid growth and have completed a higher number of fissions: results on mating inhibition differential time, between the type I and type II populations, treated with trypsin (Crippa Franceschi et al. 1987), agree with the model.

A new search was planned to verify whether non-synchronized lines of the two mating types differed from each other in their total protein content, under reactivity conditions. Furthermore, is was investigated whether the expected differences in both macronuclear DNA and total protein contents would also occur in cells of mating type I and mating type II synchronized lines. It was a question of using a different culture method, besides the classical one, which could select, from both mating types populations, lines growing at similar fission rates as possible to each other. Finally, the survey was extended to the macronuclear DNA and total protein contents in cells of both mating types having the same age, and at increasing fission ages during their log-growth phase following conjugation.

MATERIALS AND METHODS

Culture methods

Paramecium primaurelia stock 90 was grown at 25°C in monoxenic lettuce medium bacterized with *Klebsiella pneumoniae* (Sonneborn 1970). After conjugation between mating type I and mating type II karyonides, the products of the first and of the second post-zygotic fission (karyonides and sub-karyonides, respectively) were isolated in depression slides; consequently, four sub-karyonides were obtained from one reorganized cell and these were allowed to originate their progenies. As a rule, the two sub-karyonidal sister lines will express the same mating type (Sonneborn 1937,1939).

The two sub-karyonidal sister cells were cultivated according to two different methods. One of the cells was allowed to multiply in depression slide at 25°C for three days without any cell reisolation; then, its whole progeny was transferred into test-tube containing fresh bacterized medium. Mating type expression occurred within three days after transfer into test-tube. Therefore, mating reactivity induced according to Sonneborn's method (1950) took place early in the maturity period (Siegel 1961). The other sub-karyonidal sister cell was allowed to multiply in depression slide for five days at 22°C without reisolating cells; then, cells were cultured at 25°C by daily reisolation up to 22 fission age. In order to eliminate differences in fission rates, only cells with fission rates as similar to each other as possible were isolated from lines of both mating types. Finally, the

lines were transferred into test-tubes containing fresh bacterized medium and mating type expression occurred within three days. Therefore, mating reactivity took place late in the maturity period (Siegel 1961). During the culturing phase in depression slides, the fission rate of daily reisolated lines in a given day was equal to $\log_2$ of the number of cells derived from the individual cell isolated 24 h before. The fission age of each line on a given day was equal to the sum of daily fission rates from conjugation to that day. Consequently, the fission age of cell samples coming from lines growing in depression slides was always well determined. Instead, the fission age of test-tube growing cells was not exactly computable, therefore a rough estimate was given in this case. Cytological tests were routinely performed to detect the occurrence of macronuclear fragments characterizing the autogamic process.

At 10, 14, 16, and at about 26 fissions from conjugation, cells of both mating types growing in depression slides by daily reisolation and in test-tubes, respectively, were synchronized as for their cell cycle. Dividing cells were isolated and their daughter cells were allowed to grow for 1 h in bacterized medium before being transferred onto the slide.

The mating type of reactive cells was identified by the ability of giving mating reaction with 'tester' karyonides of stock P mating type I or of stock 90 mating type II. The sampling was made by means of the 'split-pair' method (Hiwatashi 1951). Cells of mating type I and of mating type II were mixed with neutral red stained mating type II and mating type I testers, respectively. Just formed pairs were split and the unstained partners were transferred onto the slide and allowed to dry in the air.

Cytochemical and cytofluorometric procedures

Macronuclear DNA and cell proteins were stained with Acriflavine 0.01% and Primulin 0.01 mM fluorochromes (Aldrich Chemical Company Inc.), respectively, according to the 'two-colour' method (Cornelisse and Ploem 1976, Rasmussen and Berger 1982). The mating type I and mating type II cell samples of 10, 14, 16, 26 fission ages from conjugation and 1 h from division, and the samples of 'split' cells of both mating types were fixed in ethanol-acetic acid mixture (3:1) for 20 min and hydrolyzed in 5N HCl for 45 min at room temperature. The cytofluorometric measurements were carried out as described (Delmonte Corrado and Margallo 1991),by means of a Leitz MPV II microphotometer (Leitz, Wetzlar, Germany) equipped with incident light fluorescence system (Ploem 1967, Böhm and Sprenger 1968). The fluorescence intensities of Primulin and Acriflavine were measured at 430 and 610 nm, respectively, and values were expressed in fluorescence units (FU).

RESULTS

First of all, it should be pointed out that the attempt to eliminate the difference in fission rate of the lines of the two mating types failed to be completely successful (Fig.1). Synchronization of the fission rates of the two types lines was not perfectly achieved; as a matter of fact, mating type I samples were generally
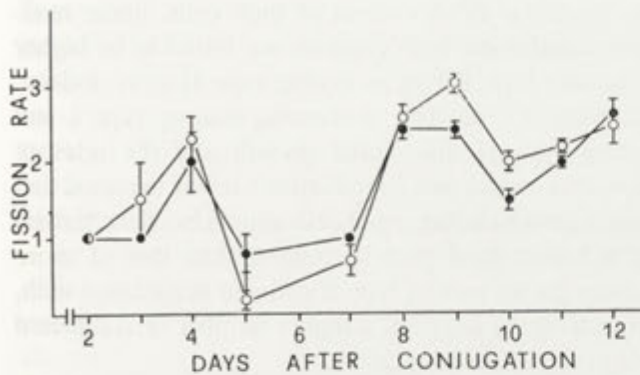
Fig. 1. Mean daily fission rate of mating type I (●) and mating type II (○) cell lines during their growth in depression slides. Cells are grown for seven days at 22°C without reisolation. From the 7th day following conjugation, they were transferred to 25°C and cultured by daily reisolation, in order to synchronize them (sample size: 20 to 35 cell lines). Vertical bars represent standard error of the means

available later than the mating type II samples of the same fission age. This is consistent with the results concerning more rapid growth of mating type II cells as compared to those of mating type I (Crippa Franceschi 1981, 1987).

The cytofluorometric measurements of 'split' cells of non-synchronized lines show significant differences in the total protein contents of the two mating types ($P < 0.05$); therefore, under reactivity conditions, mating type II is characterized by a lower total protein content, as well as by a lower macronuclear DNA content ($P < 0.01$), than mating type I (Table 1). Both contents are correlated in mating type I and in mating type II populations ($P < 0.001$ and $P < 0.01$, respectively). On the other hand, when lines of the two types growing at similar fission rates as possible to each

other (synchronized lines) are selected, the differences in their macronuclear DNA content and in their total protein content are not significant, under reactivity conditions ($P > 0.5$ and $P > 0.1$, respectively); anyway, both contents of mating type II are found to be lower than the corresponding contents of mating type I (Table 2). In this case too, macronuclear DNA and total protein amounts are correlated both in mating type I ($P < 0.01$) and in mating type II ($P < 0.001$) populations.

Comparing the cell contents of non-synchronized and synchronized lines of the same mating type, under reactivity conditions, significantly lower values in the latter than in the former case are observed for both contents (see Table 1 and Table 2). It is clear enough that sub-karyonidal sister lines of the same mating type allowed to become reactive early or late in their maturity period (see Materials and Methods) show different macronuclear and total protein contents; moreover, higher or lower contents characterize cells which completed a lower or a higher number of fissions from conjugation, respectively. Therefore, it can be assumed that, as maturity proceeds, a decrease both in macronuclear DNA and in total protein contents appears in mating reactive cells.

Fig. 2 shows the macronuclear DNA and the total protein contents of daily reisolated cells from synchronized lines of the two mating types, during their log-growth phase. A general decrease in both contents takes place in mating type I and in mating type II cells as well, between 10 to 26 fissions following conjugation. Similar trends of macronuclear DNA and total protein content values are found in both types cells; however, some mean values of mating type I cells seem to differ from those of mating type II cells. Results of the analysis

Table 1

Total protein and macronuclear DNA contents of mating type I and mating type II reactive cells of non-synchronized lines

	Mating type I cells	Mating type II cells	t^a
Total proteins[b]	22.78 ± 0.79 (53)	20.41 ± 0.71 (34)	2.07*
DNA[b]	28.34 ± 0.63 (47)	25.42 ± 0.77 (36)	2.96**
r^c	0.54***	0.42**	

[a] Student's t.
[b] Mean contents in fluorescence units ± standard error. Numbers in brackets are sample sizes.
[c] Correlation coefficient between total protein and macronuclear DNA contents.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Table 2

Total protein and macronuclear DNA contents of mating type I and mating type II reactive cells of synchronized lines

	Mating type I cells	Mating type II cells	t^a
Total proteins[b]	16.36 ± 0.68 (20)	15.02 ± 0.52 (26)	1.58
DNA[b]	20.46 ± 0.70 (20)	19.82 ± 0.81 (25)	0.57
r^c	0.59*	0.63**	

[a] Student's t.
[b] Mean contents in fluorescence units ± standard error. Numbers in brackets are sample sizes.
[c] Correlation coefficient between total protein and macronuclear DNA contents.
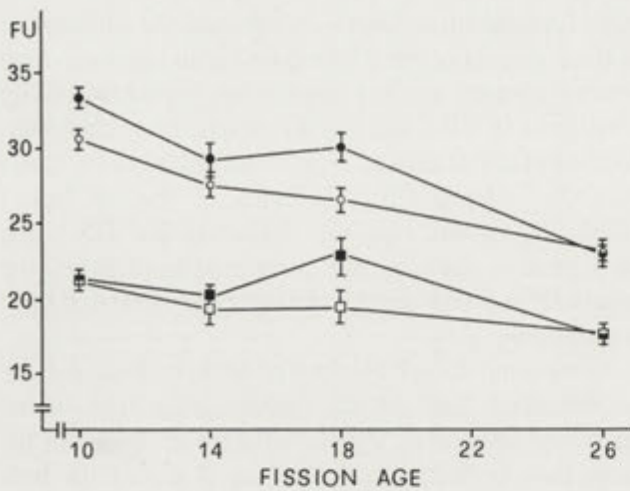*, $P < 0.01$; **, $P < 0.001$

Fig. 2. Macronuclear DNA content of mating type I (●) and mating type II (○) synchronized cells and total protein content of mating type I (■) and mating type II (□) synchronized cells, growing in log-phase. Vertical bars represent standard error of the means. FU = fluorescence units. Mean sample size = 23 cells

of variance indicate that: (i) the macronuclear DNA varies significantly regarding both fission age (P < 0.001) and mating type (P < 0.001), the interaction between the two variation sources being significant (P < 0.05; Table 3); (ii) the total protein content varies significantly regarding both fission age (P< 0.001) and the interaction between fission age and mating type (P < 0.01; Table 4).

DISCUSSION

The first evidence provided by this paper is that, when mating type I and mating type II cell lines of *Paramecium primaurelia* are allowed to develop their different growths from sexual reorganization to reactivity (non-synchronized lines), significant differences

occur in the total protein content as well as in the macronuclear DNA content of their cells, under reactivity conditions; both contents are found to be higher in mating type I than in mating type II cells. Indeed, according to the data concerning mating type I and mating type II differential growth and the relevant molecular model (see Introduction), it was expected that slowly grown mating type I cells should be characterized by a higher total protein content than that of more rapidly grown mating type II cells, in accordance with, respectively, a lower or a higher number of completed cell cycles from conjugation.

The second point concerns the relation between the culture method of the cell lines of the two mating types and the differences in their macronuclear DNA and total protein contents found under reactivity conditions. When the culture method allowed the lines of the two mating types to develop their different modes of growth (non-synchronized lines), significant differences appear both in the macronuclear DNA and in total protein contents of their 'split' cells, both values of mating type I being higher than those of mating type II. On the contrary, lines of the two mating types grown at selected fission rates (synchronized lines) do not show any significant difference either in the macronuclear DNA and in total protein contents of their 'split' cells; nevertheless, higher values are found in mating type I cells than in mating type II ones.

The last finding deals with synchronized cell lines of the two mating types, examined during their log-growth phase following conjugation. The variation of both macronuclear and total protein contents, regarding the interaction between fission age and mating type, indicates that neither the difference in macronuclear DNA contents nor the one in total protein contents between the mating types are uniform in time. This means that log-phase growing cells of the same fission

Table 3

Analysis of variance of the macronuclear DNA content of mating type I and mating type II log-phase growing cells of 10,14,16 and 26 fissions after conjugation

Source of variation	DF[a]	F
Fission age	3	87.52**
Mating type	1	12.06**
Interaction	3	3.33*

[a] Degrees of freedom
*, P < 0.05; **, P < 0.001

Table 4

Analysis of variance of the total protein content of mating type I and mating type II log-phase growing cells of 10,14,16 and 26 fissions after conjugation

Source of variation	DF[a]	F
Fission age	3	23.27**
Mating type	1	1.37*
Interaction	3	3.97*

[a] Degrees of freedom
*, P < 0.01; **, P < 0.001

age, belonging to different mating types, may differ from each other both in their macronuclear and total protein contents, between 10 to about 26 fissions after conjugation. Failure to synchronize thoroughly the two mating types, provides further evidence that mating type I and mating type II cell lines develop different modes of growth anyway (Crippa Franceschi 1981, 1987).

In conclusion, the results of this paper indicate that differences both in total protein and macronuclear DNA contents between the mating types of *P. primaurelia* are shown not only under reactivity conditions, but also during the log-growth phase of their cells. Mating types appear as cell lines growing with different physiology outlined by their different fission rates, related, in turn, to differential amounts of macronuclear DNA and total proteins.

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ACTA
PROTOZOOLOGICA

DNA Amounts in the Nuclei of Vegetative Cells and Cysts and Nucleic Acid Synthesis in Hibernating Specimens of the Ciliate *Nyctotherus cordiformis* Stein

M. N. GOLIKOVA, G. V. SELIVANOVA and V. K. NILOVA

Laboratory of Cytology of Unicellular Organisms and Laboratory of Cell Morphology, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia

Summary. The DNA content was measured (in arbitrary units, a.u.) in the nuclei of the ciliate *Nyctotherus cordiformis* out of the colon of frogs in spring, autumn and winter. The amounts of the Feulgen-DNA complex were measured with a two-wave microcytophotometer MCFU - 1. The average content of the DNA is shown to range from 1.8 a.u. in micronuclei (Mi) of cysts to 4.6 a.u. in Mi of ciliates out of the hibernating frogs. The amount of DNA in the presynthetic Mi (G_1) is supposed to be 1 - 1.5 (~ 1.25 a.u.) and in the postsynthetic Mi (G_2) 2.5 - 3 a.u. Using nuclei of the erythrocytes of *Rana esculenta* as internal standard the DNA content of a 2 c Mi of *N. cordiformis* is supposed to amount to 0.6 pg or $340 \cdot 10^9$ daltons.
The quantity of DNA in macronuclei (Ma) of *N. cordiformis* is at average 140 fold that in the Mi of cysts, the ratio rising to 600 in the case of Ma and Mi of the "winter" ciliates. The bulk content of Feulgen-DNA in Ma ranges from 260 a.u. in cysts to 2500 a.u. in "winter" ciliates.
The synthesis of the nucleic acids is demonstrated in Ma of *N. cordiformis* out of the hibernating frogs at 4°C in test tubes with the object of showing the possibility of such synthesis during the period of amphibian hibernation.

Key words. *Nyctotherus cordiformis*, quantitative cytophotometry, autoradiography.

INTRODUCTION

The complex life cycle of the ciliate *Nyctotherus cordiformis* inhabiting the colon part of the intestine of frogs and toads is closely connected to the biology of their hosts (Bojewa-Petruschewskaja 1933; Wichterman 1937; Golikova 1963, 1965). As the tadpoles differ from adult frogs, so the ciliates inhabiting tadpoles significantly differ in their morphology and cytochemistry from the cells inhabiting adult frogs. The life cycle of the

ciliate *N. cordiformis* ends by shedding off enormous quantities of cysts by adult amphibians in water basins in early spring (the period of breeding). The cysts wait at the bottom of ponds for the tadpoles to hatch. While feeding, tadpoles engulf cysts together with bottom detritus and the life cycle of *N. cordiformis* steps in its first stage. After excystment, the ciliates undergo several divisions to increase their number and turn to the stage of preconjugants.

Conjugation of *N. cordiformis* takes place at the beginning of the metamorphosis of tadpoles, corresponding to the appearance of the front pair of legs. This period of amphibian morphology is characterized by a striking phenomenon of reorganization of the whole

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intestine which transforms from the long algae-feeding type to the short carnivorous type. Apparently, the whole complex of phenomena associated with this process induces the start of the conjugation of the ciliates.

After separation of the conjugants, the ciliates pass over to the stage of exconjugants, growing together with the growth of their Ma-anlage. As a rule, the beginning of this stage corresponds to the development of the hind pair of legs in tadpoles. The enormous developed exconjugants are often found in small frogs, sometimes rather far from water basins.

The reconstruction of the definitive nuclear apparatus takes place in the intestine of small frogs, and regular reproduction of the ciliates starts the new population of *N. cordiformis* which lasts as long as the life of the host.

The present investigation shows the DNA contents in both Mi and Ma of cysts and vegetative cells of *Nyctotherus cordiformis* at different periods of their life cycle. The previous autoradiographic investigations (Golikova and Nilova 1967, 1985) demonstrated the dynamics of nucleic acids in the Ma-anlage and the adult Ma of this ciliate in the warm season. Now, along with DNA measurements in the nuclei of "winter" ciliates, an autoradiographic study was performed of the DNA and RNA synthesis in the Ma of *N. cordiformis* obtained from hibernating frogs.

A parallel measurement of the DNA amounts in the nuclei of erythrocytes of host amphibia, by the same methods and in the same arbitrary units, gave us the possibility of calculating also the absolute DNA amounts in the nuclei of *N. cordiformis*.

MATERIALS AND METHODS

The ciliates were collected from intestines of adult frogs (*Rana temporaria* and *R. esculenta*) in early spring, autumn (November) and winter (early February). For cytophotometry the ciliates were washed in Ringer's solution, fixed with ethanol-acetic acid (3 : 1), stained after Feulgen (hydrolysis at 60°C in 1N HCl for 6 min). The amount of the DNA-fuchsine complex in the nuclei was measured by the aid of a two-wave cytophotometer MCFU-1. To minimize the apparature mistakes, monochromatic light with the wave length 580 nm was used for the Mi, and 650 nm for the Ma. The relative specific coefficients of extinction ($K_{580} = 1$ and $K_{650} = 0.27$) were determined from the normalized spectrum of extinction of stained DNA-fuchsine, registered on the same cytophotometer.

Such a method allowed us to obtain optimal accuracy in photometry of nuclei (Mi and Ma) which strongly differed from one another in the concentration of the light-absorbing substance. Also the extinction of the cytoplasm was taken into account. The amount

of the DNA-fuchsine complex (in arbitrary units) was calculated according to the formula:

$$Q = \frac{(D_n - D_c) S}{K}$$

The optical density (D) was measured by scanning the background cytoplasm and the nuclei by a point light probe (4 μm in diameter). The optical density of the nucleus (D_n) was calculated from several measurements - from 3 to 50 depending from the dimensions of the nucleus, and the density of the surrounding cytoplasm (D_c) from 4 measurements.

The areas of the nuclei (S) were measured by planimetry of their enlarged negative images. K is the respective extinction coefficient (see above).

The amounts of the DNA-fuchsine in the nuclei of erythrocytes of *Rana esculenta* were measured on the same slides by the same methods at the wave length 580 nm and expressed in the same arbitrary units. The absolute quantity of the DNA in erythrocytes was taken from the literature (Rees, Jones 1972), Thus the absolute amount of the DNA in the 2 c of *N. cordiformis* could be calculated (transition coefficient - 0.42).

The ciliates out of the guts of hibernating frogs were used not only for photometry but also for autoradiography. We used ³H-thymidine (methyl-³H-1-thymidine) of molar activity 960 TBq/mol and ³H-uridine (5-³H-uridine) of molar activity 1080 TBq/mol. Both ³H-uridine and ³H-thymidine were diluted by Ringer's solution to 4mBq (l08 μCi) in 0.1 ml. All the manipulations connected with the preparation of the ciliates for the autoradiography were carried out in the presence of ice. The ciliates were incubated for 24 hours at 4°C with the radioactive precursors in 1 ml of the medium. Washed ciliates were then fixed by Carnoy's fluid, embedded in paraffin(the celloidin - paraffin embedding after Peterfi 1921 was used. Thin sections (5 μm) were prepared. Some sections were treated with either DNAse or RNAse for 3 hours at 37°C as the controls. Then, all the sections were coated by fluid emulsion (M - type or Ilford L4). The emulsions were diluted with water 1:3. The exposition was 30 days at 4°C. Autoradiographs were developed in amidol developer for the M - type emulsion (Zhinkin 1959) and in D - 19 for the Ilford L-4 emulsion. Developing time 3 min. Then the sections were stained by methyl green-pyronine and mounted in Canada balsam.

RESULTS

The great difference in the size and intensity of cytochemical reactions in the macronucleus (Ma) and the micronucleus (Mi) of *N. cordiformis* makes it often very difficult to identify the Mi in the cell. Precystic spring stages of the ciliates (Fig. l A - C) are the most convenient for the search of Mi because of the clearing of the cytoplasm which is caused by the disappearance of most food vacuoles. So, most of the measured Mi were from precyst specimens of *N. cordiformis* - 103 (Table 1). The average quantity of Feulgen-DNA in
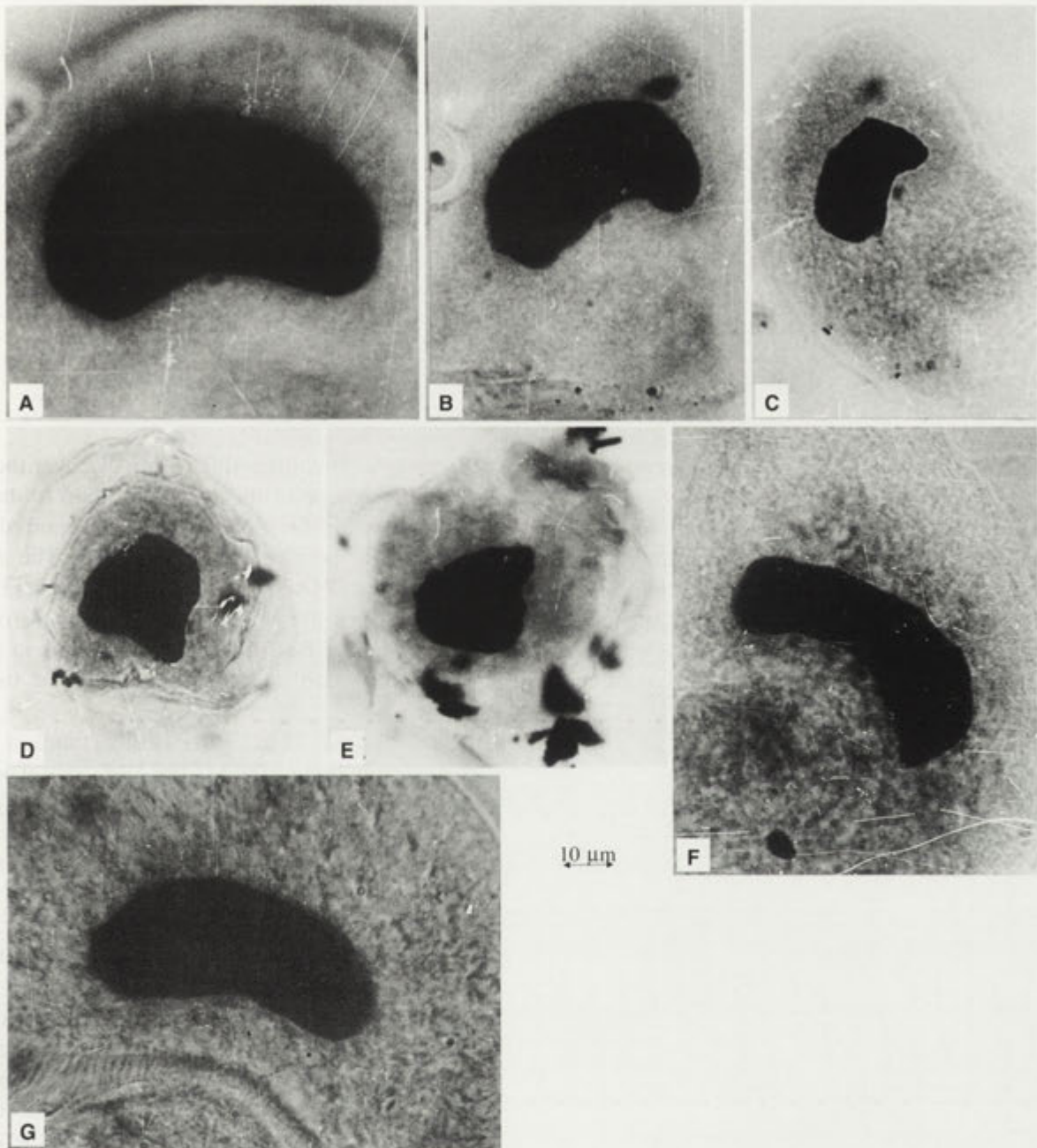
Fig. 1. Nuclei of *Nyctotherus cordiformis* at different stages of their life cycle: A - vegetative ciliate; B, C - precystic ciliates; D, E - cysts; F, G - vegetative ciliates with unusual amount of micronuclei

their Mi amounts to 3.2 ± 0.1 arbitrary units (a.u.), the values ranging from 0.7 to 6.3. Nearly 50% of all the measured nuclei were within the limits of 2 to 4 a.u. (Fig. 2 A). Some 25% of Mi of the "spring" ciliates had lower amounts of the DNA (from 0.7 to 2 a.u.) and the same percent of cells gave higher figures (from 4 to 6 a.u.).

The variability of the content of Feulgen-DNA in the Ma of "spring" ciliates (from 280 to 3000 a.u.) considerably exceeds the variation observed at other stages of *Nyctotherus* life cycle (Table 1, Fig. 3 A). This is only natural, for in spring the ciliates are rapidly dividing before the encystment and their size, as well as the size of their Ma, consequently decreases. It can be seen that

Table 1

Amounts of DNA in the nuclei of cysts and vegetative ciliates Nyctotherus cordiformis in spring, autumn and winter (in arbitrary units).

Nuclei	Micronucleus			Macronucleus			
Stages of the life cycle	Number of nuclei	DNA content Average	Range	Number of nuclei	DNA content Average	Range	Ratio Ma/Mi
Beginning of the hibernation (autumn)	38	2.8 ± 0.2	0.6 − 8.0	20	833 ± 89	405 − 1786	300
Hibernation (winter)	81	4.6 ± 0.2	1.3 − 9.0	49	2723 ± 149	1298 − 4191	600
Precystic ciliates (early spring)	103	3.2 ± 0.1	0.7 − 6.3	103	1194 ± 53	287 − 2910	375
Cysts (spring)	48	1.8 ± 0.1	0.6 − 5.0	117	258 ± 12	45 − 1054	140

nearly 30% of all Ma have an average amount of the DNA somewhere around 1250 a.u.

Enormous quantities of cysts of *N. cordiformis* are discharged from intestines of the frogs into water in early spring, during spawning time (Fig. 1 D, E). The diameters of the cysts and their Ma are correspondingly around 30 and 20 μm. The Ma loses its bean shape characteristic of vegetative ciliates and becomes nearly

Fig. 2. Amounts of DNA in the micronuclei of *Nyctotherus cordiformis* in arbitrary units: A - precystic ciliates; B - cysts; C - the beginning of hibernation (autumn); D - the end of hibernation

oval or round. It is often difficult to discover the Mi in every cyst because of its close apposition to the more brightly stained Ma. So only 48 Mi occurred to be convenient for probing out of 117 cysts. The average quantity of the DNA in them was 1.8 ± 0.1 a.u. (Table 1) the most part of the Mi being in the interval from 0.5 to 2 a.u. (Fig. 2 B). Twice as much DNA in the Mi (from 2 to 4 a.u.) was present in 23% of the cells examined.

The histogram (Fig. 3 B) demonstrates a nearly normal distribution of the Ma DNA of cysts. The mean quantity of the DNA in the Ma of 117 cysts (Table 1) is about 260 a.u., the majority of the Ma having no more than 500 a.u. The calculation shows that the Ma of the cysts contain 140 times more DNA than the Mi (Table 1).

The amphibians hibernate at the bottom of some water basins or other convenient places while having plenty of remnants of insects only partly digested in the hind parts of their intestines, and this can serve as food supply for bacteria, flagellates, amoeba, opalines and ciliates. The quantity of the DNA was measured in 20 ciliates from "autumn" frogs, but 30% of the ciliates had 2 Mi and 3 Mi (Fig. 1 F, G). Therefore the number of measured Mi became 38. The average amount of the DNA in "autumn" ciliates was 2.8 ± 0.2 a.u. (Table 1). It can be seen (Fig. 2 C) that most part of the Mi is in the range between 1.5 and 3 a.u. (60%) - 2.2 ± 0.1 a.u. at average. 29% of all the Mi in autumn have less than 2 a.u. of Feulgen-DNA and 13% - from 4 to 6 a.u.

The amount of Feulgen-DNA in the Ma of "autumn" ciliates ranges from 400 to 1800 a.u. (near 830 at average). Fig. 3 C shows that classes 400 to 500 a.u. and 1000 to 1100 a.u. exceed in frequency the other ones.
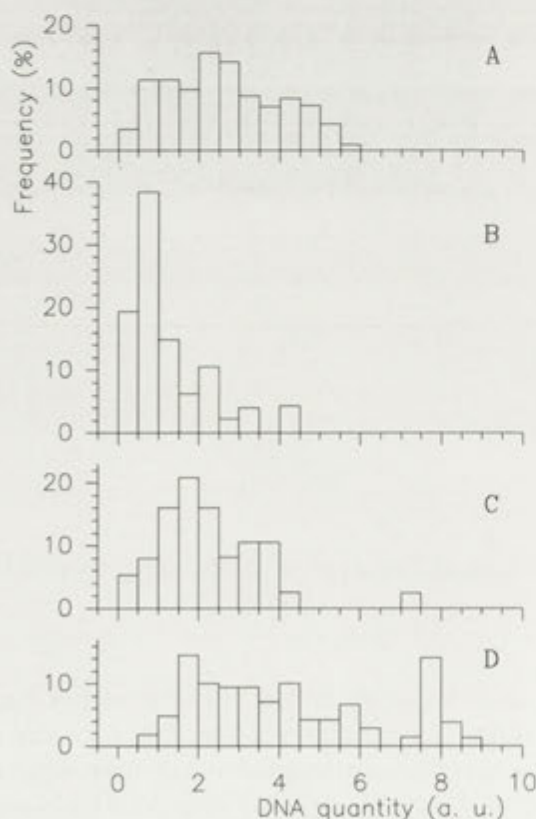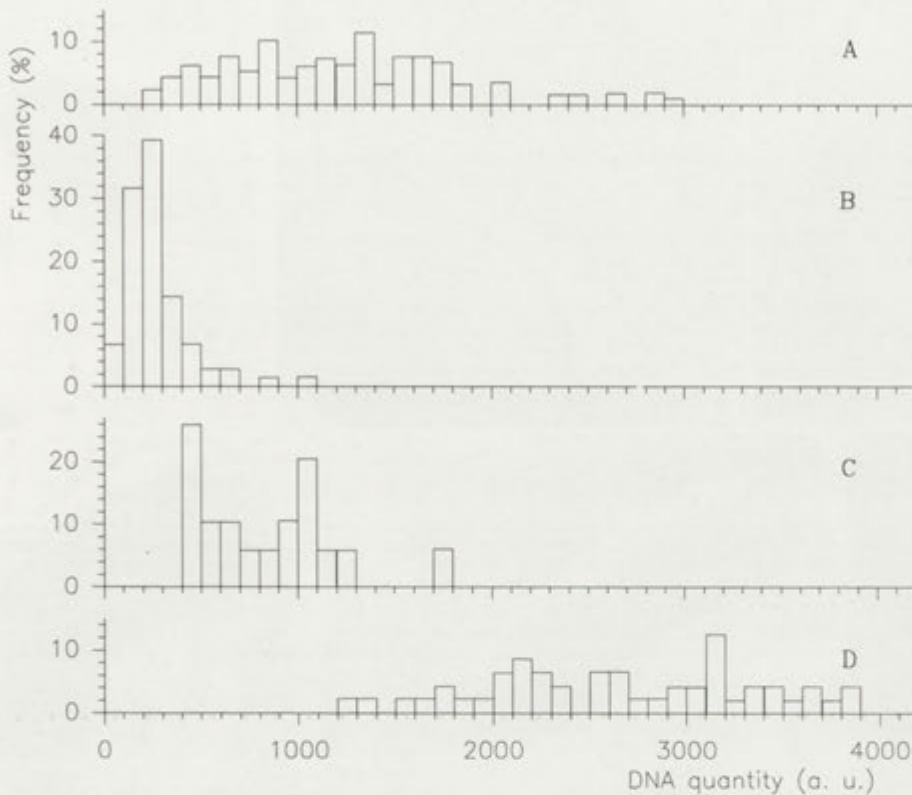
Fig. 3. Amounts of DNA in the macronuclei of *Nyctotherus cordiformis* in arbitrary units: A - precystic ciliates; B - cysts; C - the beginning of hibernation; D - the end of hibernation

The quantity of the Feulgen-DNA was measured in nuclei of 49 ciliates from the intestines of hibernating frogs (Table 1). 26 specimens of them had 1 Mi (65%), 15 (30%)- 2 Mi, 7 (15%) - 3 Mi, and one of the ciliates even had 4 - Mi. Totally, the amount of Feulgen-DNA was measured in 81 Mi. The average quantity of DNA was higher than in the Mi of other life cycle stages: 4.6 ± 0.2 a.u. (Table 1). The histogram shows a nonsignificant increase in the interval 1.5 to 3 a.u., corresponding to the maximum of the amount of DNA in the Mi of "autumn" ciliates (Fig. 2 C and D). Another small peak is within the range of 8 - 9 a.u.

The average amount of Feulgen-DNA in the Ma of "winter" *N. cordiformis* (2700 a.u.) is significantly higher than that in the Ma at other stages of the life cycle (Table 1), though the histogram demonstrates a broad variation of data with only small peaks in the intervals 2000 to 2300 a.u. and 3100 to 3200 a.u.

During autopsy, some erythrocytes have got on the slides with *N. cordiformis* from the colon of hibernating frogs. The amounts of DNA in their nuclei were measured together with the nuclei of ciliates and by the same methods (Fig. 4). The considerable dispersion of the data (from 15 to 73 a.u.) may be explained by partial destruction and digestion of the erythrocytes in the contents of the intestine prior to washing and fixation. The mean quantity of the Feulgen-DNA in the nuclei

Fig. 4. Amounts of DNA in erythrocytes of frogs (*Rana esculenta*) in the same arbitrary units
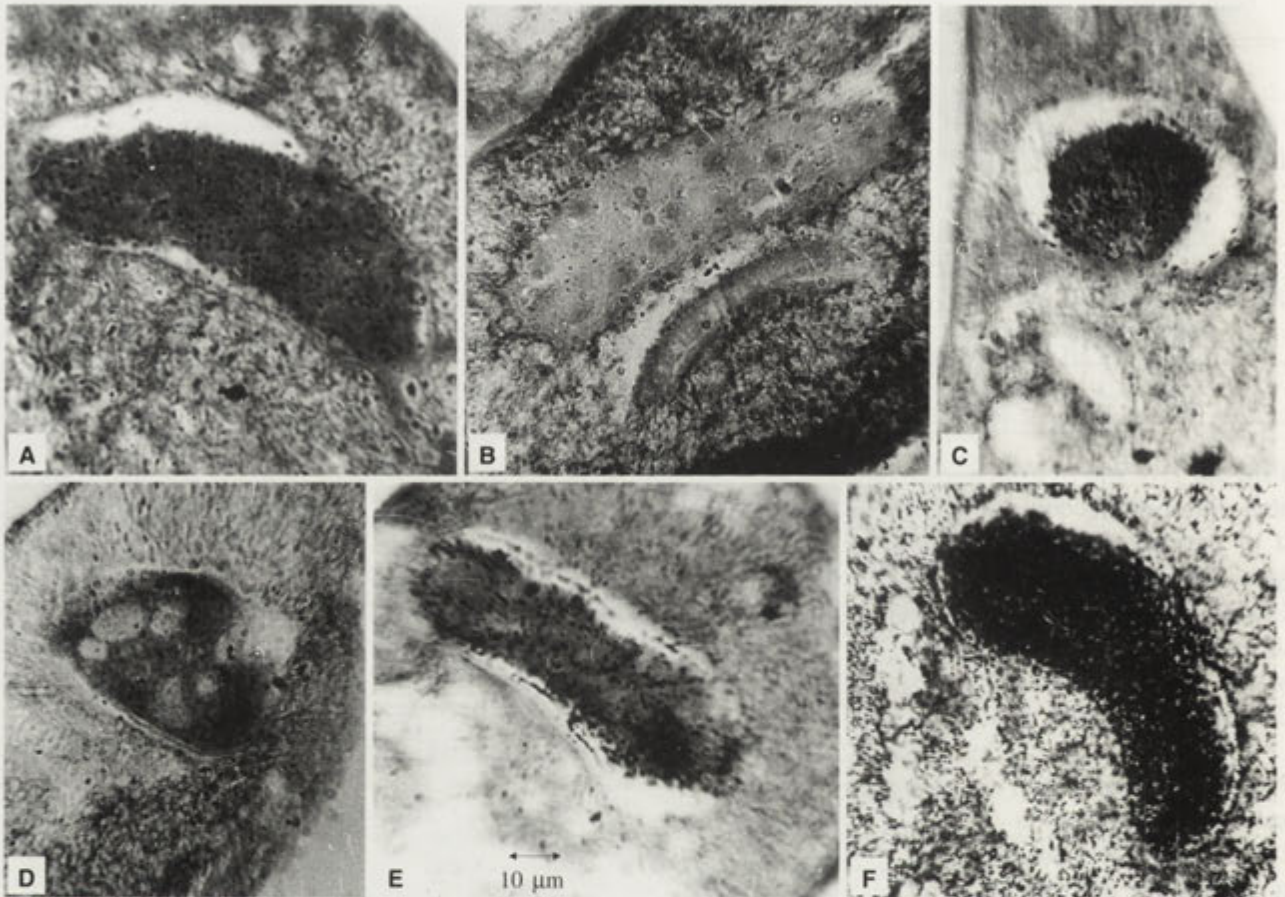
Fig. 5. Autoradiographs of sections of *Nyctotherus cordiformis*: A - the [3]H - thymidine incorporation after the 24 hours of incubation; B - the same with pretreatment of sections with DNAse; C, E - the ^{3}H-uridine incorporation; D - the same after the pretreatment of sections with RNAse; F - the result of 6 days incubation of ciliates with ^{3}H-thymidine

of erythrocytes of *Rana esculenta* in the present investigation was 40 ± 3 a.u.

Some part of the "winter" *N. cordiformis* was used for an autoradiographic investigation of the DNA and RNA synthesis in the Ma at 4°C. The autographs (Fig. 5 A) of the slides of the ciliates incubated with the ^{3}H-thymidine demonstrate a rather active synthesis of the DNA in the Ma of *N. cordiformis* at low temperature - the average temperature of amphibian hibernation. The prolonged incubation (6 days) shows considerable increase in labelling (Fig. 5 F). The pretreatment of the slides with a DNAse shows the sections to be clean of silver grains over the Ma (Fig 5 B). The results were the same in the case of incubation of the slides with ^{3}H-uridine. A rather significant amount of silver grains can be seen over the Ma (Fig, 5 C). The comparison

of pictures (Fig. 5) permits to notice that if the grains of silver are spread more or less evenly over the Ma (with the exception of nucleoli) after the incubation of ciliates with ^{3}H-thymidine, the grains are often seen in clusters after the ^{3}H-uridine incubation. Sometimes the grains are gathered around the Ma (Fig. 5 E). After the treatment of slides with the RNAse the silver grains are absent over the sections of the Ma (Fig.5 D).

DISCUSSION

The specific interest of the quantitative correlation of the DNA amounts in the nuclei of *Nyctotherus cordiformis* at different stages of the life cycle is based on the striking complication of this life cycle, including

changes of the nuclear apparatus. As the investigation was carried out on natural populations of ciliates lacking synchronization of the cell cycles, the results obtained demonstrate only the average quantities of DNA in the nuclei at different stages of the life cycle.

Table 1 shows the data on the amounts of Feulgen-DNA in both Ma and Mi in arbitrary units at various stages of the life cycle of *N. cordiformis* (cysts, precystic ciliates, vegetative cells in the beginning and at the end of host hibernation). The variation of the amounts of DNA proved to be considerable in both nuclei (Table 1, Figs 2, 3); some of the Mi happened to contain 8 times more DNA than some others. The difference between the smallest and the largest DNA figures was the least in the case of the Mi of cysts (Fig. 2). The mean quantity of the DNA in them was 1.8 a.u. (extremes: 0.6 to 5.0), the modal class being however 1.25 (Fig. 2 B). The corresponding groups of the Mi can be seen at the histograms of the precystic and "autumn" Mi (Fig. 2 B and C). Comparing all the data on the DNA contents in Mi (Table 1, Fig. 2), one can suppose this amount (1.3 a.u.) to be near the diploid presynthetic level. The presynthetic condition of the Mi of cysts seems to be energetically reasonable, the more so as the comparable spreading stages (the "swermers") of the life cycle of another ciliate, the fish ectoparasite *Ichthyophthyrius multifiliis*, have their Mi in presynthetic condition (Uspenskaja and Ovchinnikova 1966).

Then, naturally, the DNA content of the Mi in the range of 2.5 to 3 a.u. may correspond to the diploid postsynthetic level. And in fact the analysis of the histogram characterizing for example the amounts of DNA in the Mi of "autumn" ciliates (Fig. 2 C) shows a distinct domination of the group of micronuclei with the DNA content in the range of 1.5 to 3 a.u. (60% of all the nuclei). It seams that the ciliates nearly ceased to divide in a late autumn, with the environmental temperature decreasing, the largest part of the Mi being arrested in G_2. Also half of the Mi of "spring" and "winter" ciliates (Fig. 2 A, D) get in the same interval of DNA amounts (2 - 4 a.u.).

The existence of a group of Mi containing less DNA than the conventional presynthetic level (Fig. 2, Table 1), especially prominent in cysts (Fig. 2 A), permits to suppose that they are either hypoploid (a result of some abnormalities of mitosis) or simply degenerating, e.g. in non-viable cysts or cells.

Along with the defective Mi, there are some micronuclei in the cysts which contain more than the presynthetic quantity of DNA (Fig. 2 B), indicating the possibility that some of the nuclei synthesize DNA before the excystment.

The histogram (Fig. 2) permits to see that, apart from Mi of the cysts, many Mi of the other stages are in the postsynthetic state (55%, 41%, 50%). But a part of the Mi measured occurred to have more DNA than can be expected for the diploid G_2 phase. A group of Mi have four times as much DNA as the presynthetic amount (13% of "autumn" ciliates and 25% of "winter" and "spring" ciliates). And among the "winter" ciliates some 14% have Mi with five or six times more DNA than in the Mi of cysts (Fig. 2). It seems likely that, in these cases, the phenomenon of low grade polyploidy or polyteny may exist. The chromosomes of the Mi may, namely, undergo more than one replication cycle without mitotic segregation of the chromatides.

The small size and weak Feulgen staining make it very difficult to distinguish between the mitotic and interphase micronuclei. But sometimes it was possible to see the early telophase with the two closely placed future Mi (Fig. 1 G, F), resembling the mitotic Mi of *Nyctotherus ovalis* (Eichenlaub-Ritter and Ruthmann 1962).

The appearance of ciliates with two or four Mi in the hibernating populations of *N. cordiformis* may apparently be explained by the division of the Mi without a cytotomy or macronuclear division, as the result of low environmental temperature (Fig. 1 F, G).

As pointed out earlier, all quantitative data in the present study were expressed in arbitrary units. Naturally it was most important to find the place of *N. cordiformis* among the other ciliates in relation to the absolute DNA amounts in its nuclei. According to the data of some authors using different methods of measurement, the DNA amounts in the micronuclear haploid genome of various ciliates fluctuate within broad limits; from $130 \cdot 10^9$ Da in the Mi of *Tetrahymena pyriformis* (Gibson, Martin 1971; Woodard et al. 1972; Allen, Gibson 1972 a,b) and nearly $180 \cdot 10^9$ Da in the Mi of *Paramecium* of the "aurelia" group (Allen, Gibson 1972 a), through $400 \cdot 10^9$ Da in Mi of *Oxytricha* sp. (Prescott et al. 1971; Lauth et al. 1976) to $4000 \cdot 10^9$ Da in *Stylonychia mytilus* (Ammermann et al. 1974). The presence of erythrocytes of *Rana esculenta* in the smears of the contents of its colon, together with the ciliates, helped to translate our arbitrary units into absolute units. The quantity of the Feulgen-DNA in the nuclei of these erythrocytes proved to be 40 ± 3 a.u. A comparison of those results with the known data on the DNA amounts in the nuclei of erythrocytes of *R. esculenta* (Reese and

Jones 1972), 16.8pg for 2 c nuclei, permitted to calculate the approximate amount of DNA in the 2c (diploid presynthetic) Mi of *N. cordiformis*. This figure (1.3 a.u.) came out to correspond to about 0.55 pg or $340 \cdot 10^9$ Da. Of course this figure ought to be considered as rather approximate because, on the one hand, the data on the amount of DNA in the Mi of *N. cordiformis* are based on nonsynchronized cells, and on the other hand, our measurements of DNA in frog erythrocytes gave a rather wide scatter of data. The amount of DNA was the same in the Mi of *N. cordiformis* from the tadpoles of *Rana temporaria* (The data are not yet published).

The two nuclei of *N. cordiformis* being highly different in their size and intensity of the Feulgen reaction, the measurements of the DNA amounts in both of them proved impossible when the same wave length (580 nm) was used. Therefore, the quantity of Feulgen-DNA in the Ma was registered at another wavelength - 650 nm. The use of the coefficient (0.27) made the data on the DNA amounts in the Mi and the Ma comparable. The limits of the DNA amounts in all the Ma in the present material proved to be very wide - from 45 to 4200 a.u. (Table 1). The least variable were the data on cell size and macronuclear DNA content in cysts of *N. cordiformis*. The ciliates are the smallest at this stage and accordingly their Ma are also the smallest (Fig. 1). The same can be applied to the amounts of DNA in their Ma - 250 ± 12 a.u. at average (Table 1). The relation between the mean amounts of the DNA in the Ma and the Mi of the cysts occurred to be around 140. But a comparison of the average quantities of the DNA of the modal classes of both nuclei (Figures 2 and 3) raises this ratio to 200 times.

The group in a way opposite to the cysts are the "winter" ciliates (Fig. 3 A - D, Table 1). Their Ma have at average 2700 a.u. of Feulgen-DNA. The histogram of the DNA amounts in those Ma is very extended (Fig. 3 D). Most nuclei have 2000 to 3500 a.u. of Feulgen-DNA. The quantity of the DNA in the Ma of the "winter" ciliates is 600 times more than in their Mi (Table 1).

There are two distinct dominant groups of Ma (from 400 to 700 a.u. and from 900 to 1100 a.u.) on the histogram reflecting the amounts of the Feulgen-DNA in the "autumn" ciliates (Fig. 3 C). The average amount of DNA in their Ma is 300 times higher than that in their Mi (Table 1), and a comparison of the modal classes gives the same figures (250-300).

Naturally the Ma of the "spring" ciliates undergoing frequent successive divisions demonstrate the most con-siderable variation in the DNA amounts (Fig. 3 A) - from 290 to 2900 a.u., 1200 a.u. on average (Table 1). The quantity of the DNA in Ma at this stage of the life cycle of *N. cordiformis* is 375 times more than the same in Mi, and the comparison of the modal classes (Fig. 2 A) gives figures from 300 to 500.

Thus the relation between the amounts of DNA in Ma and Mi is the least in the case of nuclei in cysts, this relation being higher at other stages of the life cycle. Both nuclei may be supposed to exist in presyn-thetic condition in cysts, whereas most nuclei, both Mi and Ma, at other life cycle stages are evidently in postsysthetic condition.

The autoradiographic investigation demonstrates (Fig. 5 A - F) the presence of DNA synthesis in Ma of *N. cordiformis* at low temperature (4°C) in test tubes, in the contrast to *Opalina inhabiting* the intestines of the same hosts (Nilova and Sukhanova 1969). The RNA synthesis also proved to be rather active (Fig. 5 C - E). The fact permits us to propose the possibility of func-tional activity of Ma of *N. cordiformis* in the period of hosts hibernation.

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ACTA PROTOZOOLOGICA

UV Effects on Photosynthetic Oxygen Production and Chromoprotein Composition in a Freshwater Flagellate *Cryptomonas*[*]

Andrea HÄBERLEIN and Donat-P. HÄDER

Institut für Botanik und Pharmazeutische Biologie, Friedrich-Alexander Universität, Erlangen, Germany

Summary. Photosynthetic oxygen production in the freshwater cryptoflagellate, *Cryptomonas S2*, was shown to be impaired after short exposure to solar or artificial ultraviolet radiation. Results from both polyacrylamide gel electrophoresis and fast protein liquid chromatography (FPLC) analyses indicated that the phycobiliproteins are affected readily by even short exposure, while the membrane bound chlorophyll protein complexes are hardly altered. Fluorescence excitation and emission spectra indicate that the energy transfer from the accessory pigments to the photosystems is impaired by ultraviolet radiation. Furthermore, phycoerythrin could be shown to occur in three isomers.

Key words. *Cryptomonas*, FPLC, fluorescence spectroscopy, gel electrophoresis, oxygen production, ultraviolet radiation.

INTRODUCTION

More than half of the photosynthetic biomass on our planet is produced by marine and freshwater phytoplankton (Houghton and Woodwell 1989). Cryptophyceae are among the most important groups and produce large algal blooms in the oceans and in freshwater habitats (Burns and Rosa 1980, Kamiya and Miyachi 1984, Spector 1984). Phytoplankton communities have been found to be affected by solar UV-B irradiation (280 - 320 nm) even at current levels (Worrest and Häder 1989, Häder and Worrest 1991). Any substantial increase in the solar UV component due to a partial thinning of the stratospheric ozone layer is there-fore thought to have adverse effects on the ecosystem and the biological foodweb (El-Sayed 1988).

Crytophyceae orient in their habitat by using orientation strategies which utilize external factors such as light and gravity (Nultsch and Häder 1988). In contrast to the marine *Cryptomonas maculata*, which shows positive phototaxis at low fluence rates and negative phototaxis at high fluence rates (Häder et al. 1987, 1988), and to an unidentified freshwater species which shows only positive phototaxis (Watanabe and Furuya 1974, Watanabe et al. 1976), the freshwater *Cryptomonas S2* orients by diaphototaxis (orientation perpendicular to the actinic light beam) resulting in a horizontal movement in the natural water column (Rhiel et al. 1988a,b). At low fluence rates this behavior is offset by a slight upward component which brings the population closer to the surface and at high fluence rates by a downward component which takes the cells deeper down in the water column. In addition, there is a

pronounced negative gravitaxis (Rhiel et al. 1988b) by which the cells move to the surface if not oriented with respect to other stimuli, e.g. at night.

Solar radiation has been found in field studies to impair the photoorientation of the organisms (Häder and Häder 1989c). Histograms of the direction distribution of the cells show a deterioration of the precision of movement after exposure to unfiltered sunlight even after short exposure times of 1 or 2 h. Simultaneously, the percentage of motile cells in the population decreased dramatically during exposure. Likewise, the swimming velocity of the motile fraction decreased drastically after an exposure of about 80 min. A significant part of the inhibitory action of solar radiation seems to be the UV-B component, since removal of this spectral band by UV cut-off filters strongly prolonged motility. Also filtering the radiation by an artificially produced layer of ozone decreased the inhibition. These results were supported by inhibition of orientation and motility induced by artificial ultraviolet radiation (Häder and Häder 1990). Similar inhibitory effects were observed in a number of freshwater and marine phytoplankton (Häder and Häder 1988a,b, 1989a,b,c, 1991, Häder et al. 1990a,b). In some cases also the gravitactic orientation is impaired by solar ultraviolet radiation (Häder and Liu 1990a,b).

When exposed to either solar or artificial ultraviolet radiation the photosynthetic pigments are bleached as shown by difference absorption spectroscopy (Häder and Häder 1989c, 1990). It is interesting to note that the accessory pigment phycoerythrin is bleached first, followed by the carotenoids, while the chlorophylls *a* and *c* are most resistant to UV radiation in a marine *Cryptomonas* (Häder and Häder 1991).

The aim of the present study is to investigate the effects of ultraviolet radiation on absorption and fluorescence spectra as well as the photosynthetic activity and to demonstrate the UV effects on the protein components using gel electrophoresis and FPLC.

MATERIALS AND METHODS

Organism and culture conditions

The up to now unidentified *Cryptomonas* species S2 isolated from a pond near Marburg (Rhiel et al. 1988a) was grown in static cultures in 1 l Erlenmeyer flasks containing 400 ml of a medium described previously (von Stosch 1973) at 20°C under continuous light from mixed daylight and warm tone fluorescence lamps (5 W m^{-2}).

UV irradiation

The organisms were exposed to artificial ultraviolet irradiation which was produced from a transilluminator (peak at 312 nm,

Bachofer, Reutlingen, FRG); the radiation extended from 280 to 400 nm at about 1 W m^{-2}. Cultures were split in equal halves, one of which served as a control, and the other aliquot was transferred into a flat container and irradiated for the times specified in the results section.

Exposure to solar radiation was carried out between August, 8th and 29th, 1990 at Caparica (Portugal, 38° North) on sunny days between 11.00 and 15.00 local time. During this period the UV-B irradiation amounted to about 2.9 W m^{-2}, as measured with an Optronics spectroradiometer (model 742, Optronic, Orlando, Fla, USA) by the group of Prof. Tevini. Cell suspensions were harvested by centrifugation, placed in open vessels (100 mm in diameter, 3 mm suspension layer) and exposed to sunlight in a custom-made, temperature-controlled growth chamber (Weiss, Giessen, FRG) kept at 20°C. Plexiglass roof cuvettes (transmitting 92% of the radiation between 280 and 750 nm) allowed exposure of the cell suspensions to solar radiation.

Photosynthetic oxygen production

Photosynthetic oxygen production was determined in a custom-made container which consisted of a Plexiglas cylinder with 20 mm inner diameter inside a water jacket connected with a thermostat (RMT6, Dr. Wobser GmbH, Lauda-Königshofen). Aliquots of 5 ml cell suspension were contained in the sample compartment and agitated by a magnetic stirrer. A Clark electrode (Yellow Springs Instruments, Ohio, USA) (Dubinsky et al. 1987) was connected via a custom-made polarizer (Estabrook 1967) with a recorder (PM 8262, Philips, Netherlands). Calibration was achieved with 10 mM sodium dithionite for 0% oxygen saturation and the 100% value was recorded after bubbling air through the suspension for 60 min. Photosynthetic oxygen production was induced by actinic light from a 250 W slide projector with a 24 V quartz halogen bulb (Kindermann Universal, Wetzlar, FRG). Light intensity was adjusted by inserting neutral density filters rather than the built-in dimmer, in order to keep the color temperature of the light beam constant, and measured with a thermopile (Type A 1754385; Kipp & Zonen, The Netherlands), connected to a microvoltmeter (Keithley, type 155). Light intensities inside the sample compartment were measured with a PIN diode (Optoelektronik Lasertechnik, Gröbenzell, FRG) previously calibrated against the thermopile.

Absorption and fluorescence spectra

Absorption spectra of cells and isolated protein fractions were measured in quartz cuvettes with an optical path length of 10 mm (Hellma, Müllheim, FRG, 2 mm thickness) in a DU 70 spectrophotometer (Beckman, Palo Alto, CA, USA). Fluorescence spectra of cell suspensions stabilized in 0.5% agar in the quartz cuvette were measured with a Shimadzu RF 5000 spectrofluorimeter. In addition, fluorescence spectra were recorded from protein fractions collected from the FPLC (see below).

Cell fractionation

Cells were harvested by centrifugation in a refrigerated centrifuge (J2-21 M/E) using a JA 10 rotor (Beckman Instruments) at 2740 x g for 10 min at 10°C. The pellet was resuspended in 10 ml solubilization buffer (25 mM Tris/HCl pH 7.8, 3 mM EDTA, 2.5 mM
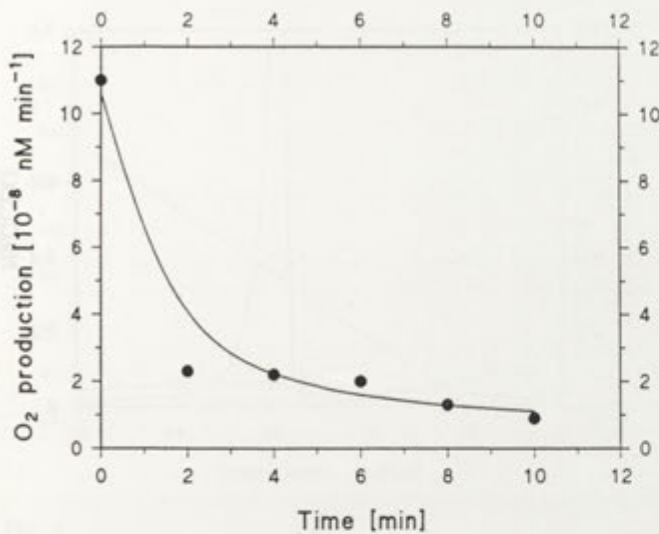
Fig. 1. Photosynthetic oxygen production per cell measured at 10 W m^{-2} white light and 25°C after increasing exposure to solar radiation (1140 W m^{-2} and a UV-B component of 2.9 W m^{-2})

Fig. 2. Photosynthetic oxygen production per cell measured at 10 W m^{-2} white light and 27°C after increasing exposure to ultraviolet radiation of 0.8 W m^{-2} (open circles) and 0.5 W m^{-2} (filled circles). Error bars indicate standard deviation for 5 independent measurements

dithiothreitol, 5 mM MgCl$_2$, 1 mM phenyl methyl sulfonyl fluoride). The cells were ruptured in a French press (Aminco, Urbana IL, USA 3 times at 1770 bar, 1 drop per s) and centrifuged 5 min at 850 x g (Sigma table top centrifuge 2 MK Osterode, FRG) to remove cell debris. The supernatant was centrifuged at 80,000 x g at 4°C for 1 h (ultracentrifuge Beckman, rotor 75 Ti). The supernatant contains most of the cytoplasmic proteins and the pellet was enriched in the membrane proteins which were solubilized in detergent buffer which contained 0.5% polyoxyethylen 10 tridecyl ether (Emulphogen) and 25 mM sorbitol in addition to the solubilization buffer described above. Solubilization was enhanced by treating the sample in a potter (Braun, Melsungen) and by sonification (Sonifier 450, Bransen, Danbury, USA) for 5 min at level 2 at a duty cycle of 20% on ice. After two more centrifugation at 80,000 and 100,000 x g for 1 - 2 h the membrane proteins were found in the supernatant.

Protein purification

Proteins were separated in 11 ml of a sucrose gradient (20 - 40% in solubilization buffer for the cytoplasmic proteins and 5 - 50% in detergent buffer for the membrane proteins). Aliquots of 0.5 - 1 ml of the sample was added and the tubes were centrifuged at 150,000 x g (4°C) in a SW 40 rotor (Beckman). Protein concentration was performed using the technique of Bradford (1976) calibrated using bovine serum albumin.

Gel electrophoresis of the proteins was carried out on a horizontal Multiphor II system (Pharmacia LKB, Bromma, Sweden) in a gradient 4 - 22.4% T on a Bond PAG film or on a vertical system (2001, Pharmacia LKB) using a 5 - 22.5% T gradient gel. Visualization of the protein bands was achieved by an enhanced silver staining (Görg et al. 1988).

FPLC separation of cytoplasmic proteins was performed on a system by Pharmacia composed of the liquid chromatography controller (LCC-500) with pH monitor, two pumps, a variable
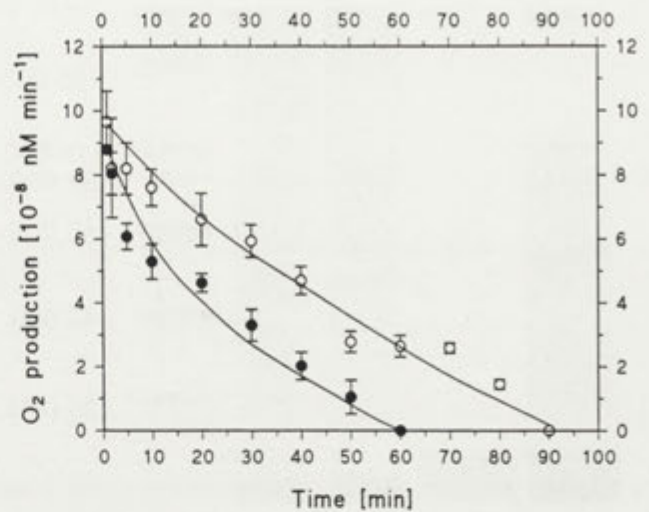
wavelength monitor and a fraction collector (Frac-100). The samples were separated on a MonoQ column, which is a strong anion exchanger, in Tris buffer (pH 8.5) with 0.1% Emulphogen added. The membrane proteins were separated on a gel filtration column (Superdex 200, Pharmacia LKB).

RESULTS

Photosynthetic oxygen production

In preliminary experiments, the conditions for photosynthesis measurements were optimized. Under the conditions used, the cells showed logarithmic growth rate between the 4th and 14th day with a doubling time of about 7 days. The chlorophyll concentration per cell (measured at 676 nm and adjusted for cell density) increased with age. At a cell density of 10^6 per ml, at 20°C, maximal oxygen production was measured at 10 W m^{-2} (data not shown), with a peak of 11 x 10^{-8} nM min^{-1} cell^{-1} corrected for respiration. The net O$_2$ production had an optimum at 10^6 cells per ml and the optimal temperature was found at 27°C while respiration peaked at 31°C (data not shown). A similar temperature optimum was found for higher C3 plants (Lawlor 1990). Photosynthetic O$_2$ production was highest in young cells and declined with age.

When exposed to solar radiation photosynthetic O$_2$ production was found to decrease rapidly even after short exposure times (Fig. 1). Likewise, photosynthesis
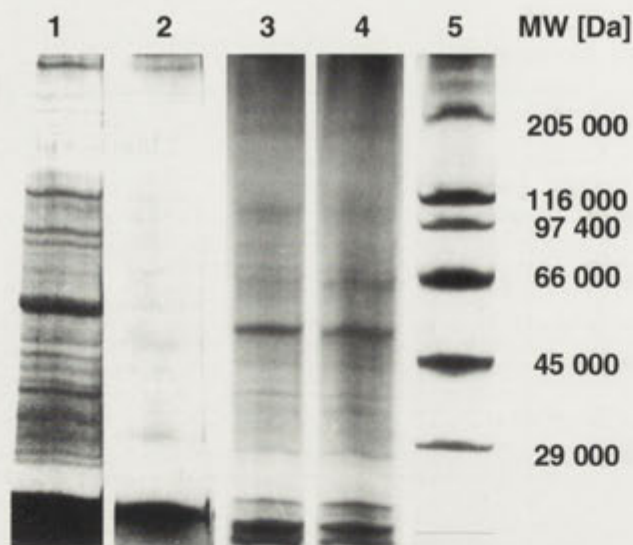
Fig. 3. Horizontal SDS PAGE (gradient: 4 - 22% T) of the cytoplasmic fraction from *Cryptomonas* S2 before (lane 1) and after 4 h of artificial ultraviolet radiation (lane 2). Membrane fraction before (lane 3) and after 4 h of radiation (lane 4) in comparison to the marker proteins (lane 5)

Fig. 4. Elution diagrams from an FPLC run on an anion exchange column (MonoQ) monitored at 280 nm (solid lines) and at 565 nm (dotted curve) before (upper curves) and after (lower curves) exposure to ultraviolet radiation for 4 h. The straight line indicates the NaCl gradient used for elution

was impaired under artificial UV irradiation (Fig. 2); lower irradiance (controlled by inserting UV transmitting neutral density filters) prolonged the tolerated exposure times.

Protein isolation

On the sucrose gradient, the cytoplasmic fraction showed a pronounced red band representing the phycobilins. After 4 h artificial UV treatment, the colored band disappeared completely. In contrast, the membrane fraction separated into two bands on the sucrose gradient; the upper one showed an intense green staining and the lower one a weak green tint. UV radiation had no visible effect on the membrane fraction on the sucrose gradient.

On horizontal SDS PAGE, the cytoplasmic fraction was separated in a number of bands with apparent molecular weights in the range between 29,000 and 205,000 (Fig. 3). After UV irradiation all bands were

Fig. 5. Elution diagrams of the membrane fraction from an FPLC run on a gel filtration column (Superdex 200) monitored at 280 nm before (solid line) and after (dotted line) 4 h of UV irradiation

Fig. 6. Absorption spectrum of a crude extract from *Cryptomonas* S2 before (upper curve) and after (lower curve) 4 h exposure to artificial ultraviolet radiation. Ordinate, absorption in O.D.
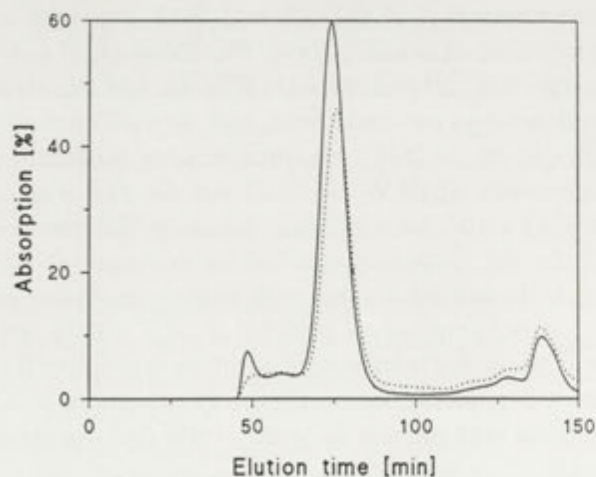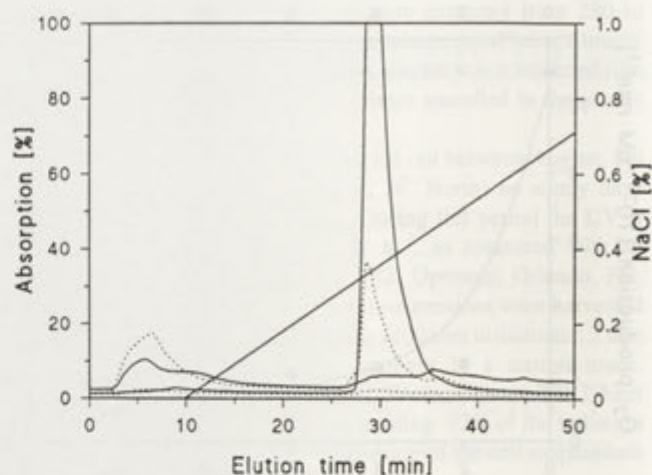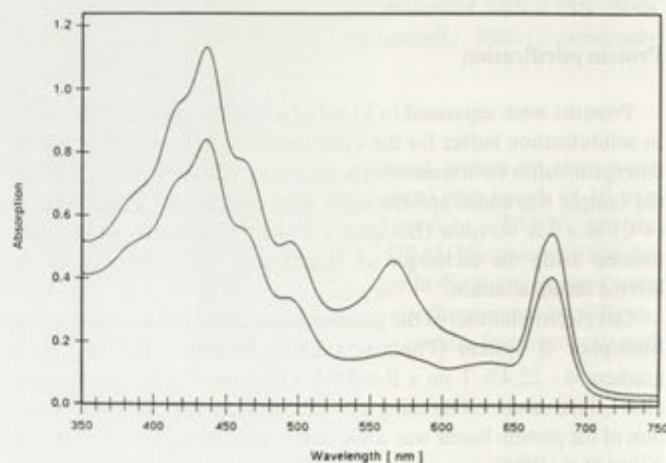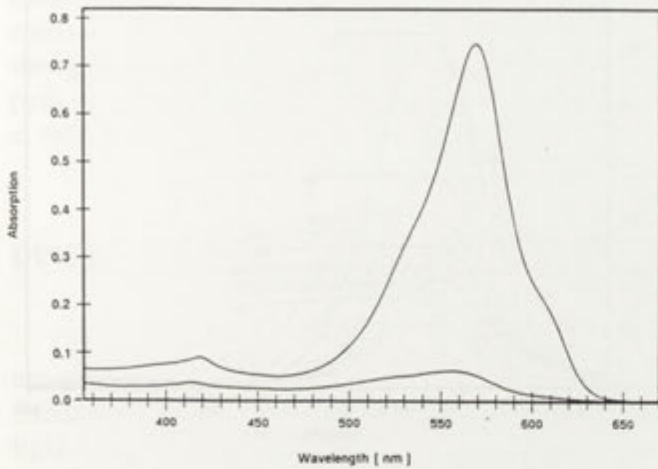
Fig. 7. Absorption spectra of the cytoplasmic fraction from *Cryptomonas* S2 before (top curve) and after (lower curve) 4 h exposure to artificial ultraviolet radiation

threshold adjusted to 5%. The elution diagram of the UV treated sample had the same appearance. When further separated on vertical SDS PAGE, fraction 1 of the membrane sample collected from the gel filtration column showed several bands in the range between 14,200 and 66,000. Fraction 2 had bands between about 10,000 and 29,000. The UV treated sample did not differ in the SDS PAGE pattern.

Absorption and fluorescence spectroscopy

The absorption spectrum of the cell homogenate showed major peaks at 436, 565 and 678 nm in addition

considerably weaker and some bands were no longer detectable. The membrane fraction showed bands in the range from below 20,000 to close to 100,000; in accordance with the sucrose gradient results this pattern was not altered by previous UV irradiation of the cells.

The cytoplasmic proteins were separated on the MonoQ ion exchange column using the FPLC (Fig. 4). The control sample produced an elution profile with three peaks, the first of which preceded the onset of the NaCl gradient and consisted of proteins not bound to the column material and had a slight yellow green tint. The second fraction eluted at 0.32 M NaCl and showed a red color. The third small peak, at 0.48 M NaCl, was too small to be collected. Also, the UV irradiated sample showed three elution peaks (before the onset of the salt gradient, at 0.3 M and at 0.5 M, respectively). None of the fractions showed a measurable absorption. The FPLC fractions were subjected to vertical SDS PAGE: fraction 1 (non bound proteins) showed several bands in the range between 14,200 and 66,000 while fraction 2 had only one pronounced band at an apparent molecular weight of 20,100. In the UV treated samples, no protein band could be detected in the vertical SDS PAGE after FPLC separation.

The membrane fraction was separated on a gel filtration column (Superdex 200) at a flow rate of 1 ml min^{-1}. Like on the sucrose gradient two major fractions could be detected (Fig. 5), a green fraction, which ran off the column at 67 min, and a colorless fraction which occurred at 120 min. Other fractions were not collected because their absorption was below the sampling

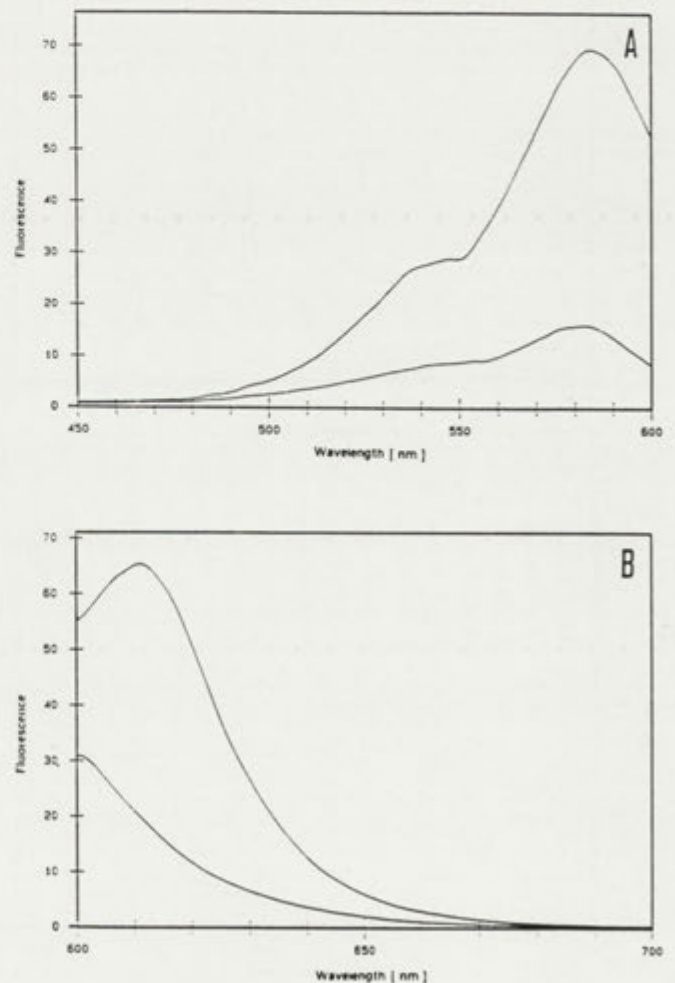Fig. 8. Fluorescence excitation spectra (a, monitored at an emission of 612 nm) and emission spectra (b, excited at 585 nm) of the cytoplasmic fraction from *Cryptomonas* S2 measured before (upper curves) and after 4 h of artificial UV radiation (lower curves)

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to a number of shoulders (Fig. 6). After 4 h UV treatment the absorption was generally decreased at all wavelengths, but specifically the peak at 565 nm, indicative of phycoerythrin, was massively bleached. The cytoplasmic fraction showed a major peak at 565 nm in the absorption spectrum which was also almost completely bleached after UV treatment (Fig. 7). The excitation spectrum, monitored at an emission of 612 nm, had a maximum at 585 nm and a shoulder at 542 nm, both of which declined sharply after UV treatment (Fig. 8a). When excited at 585 nm the fluorescence emission spectrum of the cytoplasmic fraction showed a peak at 612 nm which decreased almost completely after UV irradiation (Fig. 8b).
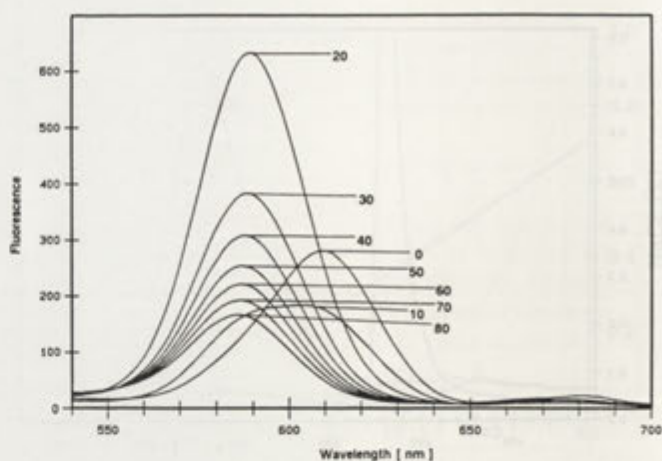
Fig. 10. Fluorescence synchronous spectra of *Cryptomonas* S2 *in vivo* after increasing exposure times to solar radiation (numbers at the curves, in min). While the excitation monochromator scanned the spectral range the emission monochromator was synchronously adjusted to wavelengths 20 nm longer. During radiation the total solar energy amounted to 1 140 W m^{-2} and the UV-B range totalled at 2.9 W m^{-2}

The absorption spectrum of the membrane fraction looked similar to the one of the crude homogenate, but the 565 nm peak was absent (data not shown). When excited at 436 nm the fluorescence emission spectrum of the membrane fraction had peaks at 606, 640 and 673 nm (Fig. 9a); after UV treatment specifically the two longer emission peaks decreased in intensity. The excitation spectrum, monitored at 673 nm had maxima at 420 and 440 nm, shoulders at 568 and 606 nm as well as a major peak at 640 nm (Fig. 9b).

When exposed to solar radiation the changes in the fluorescence spectra showed a complex behavior; this is best documented in synchronous spectra in which both the excitation and the emission monochromators scan the spectrum simultaneously; the wavelength difference was fixed in this experiment to 20 nm (Fig. 10). In addition to the very small emission near 680 nm, there was a pronounced peak at 612 nm. When measured at 10 min intervals between exposure first the emission at 612 nm decreases and a second emission peak occurs at 587 nm. After 30 min of exposure the 612 nm peak completely disappears and the 587 nm increases sharply to drop after further solar exposure. Finally a third component can be seen as shoulder arising after 40 min with an emission at 577 nm. When monitored at shorter time intervals a gradual decrease at the 612 nm emission and a gradual increase in the 587 nm emission can be observed. An interesting result was obtained when solar radiation was filtered through a long wavelength passing
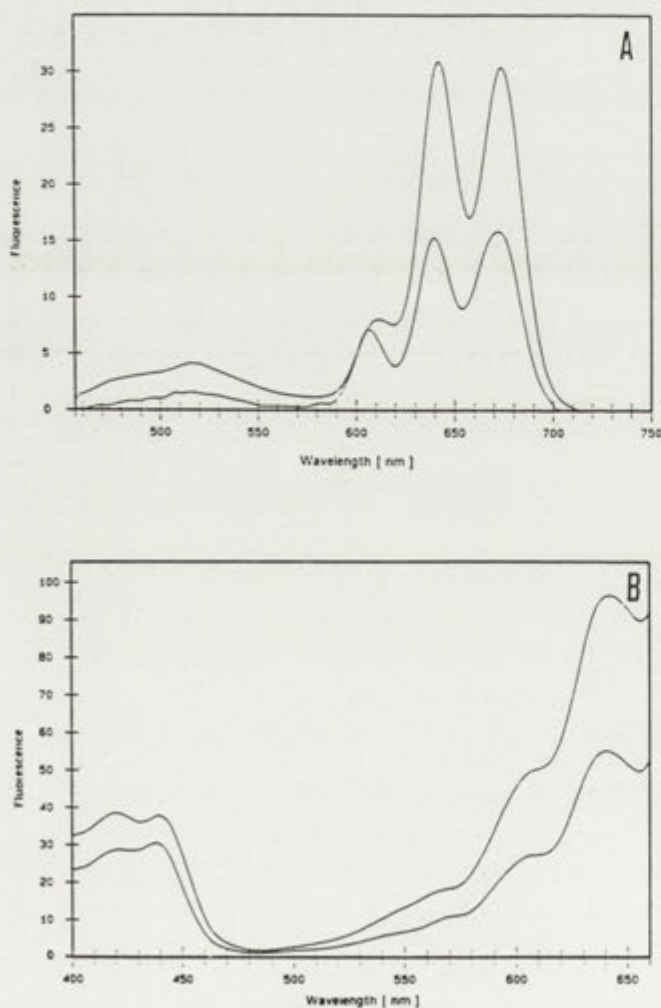

Fig. 9. Fluorescence emission spectra (a, excited at 436 nm) and excitation spectra (b, monitored at an emission of 673 nm) of the membrane fraction from *Cryptomonas* S2 measured before (top curves) and after 4 h of artificial UV radiation (bottom curves)

filter with a cut off at 495 nm (GG 495): the initial emission at 612 nm shifts to 687 nm as in unfiltered sunlight. However, after about 2 h of exposure the latter peak decreases and a new one builds up with an emission at 603 nm (data not shown).

DISCUSSION

The cryptoflagellates used in this study orient in their natural habitat using light and gravity as major clues (Häder et al. 1987, 1988) to move to a layer of suitable light conditions within the water column. The cells are exposed to considerable ultraviolet fluence rates at the depth they usually occupy, as calculated from the transmission curves of various bodies of water (Jerlov 1970, Baker and Smith 1982). Previous results have shown that even short exposure to solar or artificial ultraviolet radiation affects the pigmentation of the cells; however, with different bleaching kinetics for the photosynthetic pigments. The accessory pigments (phycoerythrin) are bleached first, while chlorophyll *a* is not impaired and chlorophyll *c* shows a partial decrease. These observations are confirmed by the present biochemical and spectroscopic results, which show that phycoerythrin mainly found in the cytosolic fraction decreases much more effectively under irradiation than the chlorophylls found in the membrane fraction. Two competing processes occur at the level of the accessory pigments during irradiation: in addition to a long term decrease in the pigment concentration there is a fast increase in the fluorescence emission indicating that the energy transmission to the reaction centers is impaired, so that more excitation energy is wasted in the form of fluorescence. In addition, there are remarkable shifts in the emission wavelength both after solar and artificial radiation. These findings support the results of Mörschel and Wehrmeyer (1977) and Hill and Rowan (1989) who demonstrated multiple forms of phycoerythrin in *Cryptomonas* absorbing at 545 nm (type I), 555 nm (type II) and 565-568 (type III), which can be separated by isoelectric focusing. Obviously, the phycoerythrin in *Cryptomonas* S2 belongs to type III which has a fluorescence emission at 612 nm. It is interesting to note that under irradiation the emission peaks significantly shift indicating a photoconversion into other phycoerythrin charge isomers. The sequential occurrence indicates that these charge isomers may be degradation products of an initial form.

Chlorophyll *a* in this organism has a Soret band at 436 nm and a red peak at 673 nm while chlorophyll *c* absorbs at 460 and 632 nm. Judging from the present absorption and fluorescence data the main component of the membrane fraction is the chlorophyll *a/c* carotenoid complex also described for diatoms (Brown 1988). Small peaks, or shoulders, in the spectrum of the cytosolic fraction indicate that it contains traces of the membrane fraction and *vice versa*.

The photosynthetic capacity of the cells, measured using a constant white test light after increasing UV exposure, declines even much faster than the changes observed in protein analysis and absorption spectroscopy indicating that structural changes within the photosynthetic apparatus are induced by rather short exposure times which affect the energy transfer within the antenna complex, while UV induced photobleaching occurs only after prolonged exposure. A similar inhibition of the photosynthetic oxygen production by UV-B irradiation was found in the diatom *Thalassiosira pseudonana* (Cullen and Lesser 1990), in which a short exposure time at high fluence rates had a more pronounced effect than a long exposure time at low fluence rates, suggesting the presence of a repair mechanism.

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ACTA
PROTOZOOLOGICA

Ectobiotic and Endocytobiotic Bacteria Associated with the Termite Flagellate *Joenia annectens*

Renate RADEK[*], Klaus HAUSMANN[*] and Alfred BREUNIG[**]

[*]Freie Universität Berlin, Institut für Zoologie, AG Protozoologie, Berlin; [**]Universität Ulm, Angewandte Mikrobiologie, Ulm, Germany

Summary. In this study the association of the hypermastigote flagellate *Joenia annectens*, symbiotic in the termite *Kalotermes flavicollis*, with various kinds of bacteria was investigated. Several kinds of non-motile ectobiotic rod-like bacteria covered most of the flagellate's body. Occasionally, spirochetes attached as well. Specialized attachment sites were formed. A curious kind of granule formation was described with the rod-like bacteria. An electron dense granule fitting the width of the bacterium was formed at the attaching cell pole. Later, the bacterial wall structures nearby invaginated thus pinching off the granule which eventually gets engulfed by the flagellate. Various types of endocytobiotic bacteria inhabited the nucleus or the cytoplasm. Spore-forming bacteria occasionally occurred accumulated in vacuoles.

Key words. Hypermastigotes, *Kalotermes flavicollis*, spirochetes, spore-formation.

INTRODUCTION

Termites and the related cockroaches harbor symbiotic flagellates of the orders Trichomonadida, Oxymonadida and Hypermastigida in their hindgut (Honigberg 1970, Radek and Hausmann 1991, 1992). Members of these flagellates are indispensable for the digestion of cellulose and possibly lignin in the xylophagous lower termites and roaches (O'Brien and Slaytor 1982). Under natural conditions all flagellates are associated with endocyto- and/or ectobiotic bacteria which may contribute cellulolytic enzymes to degrade the engulfed wood (Hungate 1955, Lavette 1969a).

Higher termites, instead, use their own enzymes and those of the dominant gut bacteria and exosymbiotic fungi which are grown by termites in their fungus gardens. Diverse protozoa such as flagellates, amoeba, gregarines and ciliates are present in higher termites but live in a non-symbiotic association (Gisler 1967).

A great variety of organisms like bacteria, fungi, viruses, algae and even protists have been described living in and on host protists (Ball 1969, Kirby 1964, Preer and Preer 1984). Some are parasites harming their host, others as for example the photosynthetic zoochlorellae and zooxanthellae are symbiots providing their hosts with nutrients. This report will focus on the bacterial association with termite flagellates. Three kinds of bacterial populations may be distinguished. Firstly, bacteria get enclosed in food vacuoles and are digested. Not only the small flagellates but also the large wood

Address for correspondence: Renate Radek, Freie Universität Berlin, Institut für Zoologie, AG Protozoologie, Königin-Luise-Str. 1-3, 1000 Berlin 33, Germany.

consuming species use this additional source of food. Other bacteria live and divide in the interior of the animal. The cytoplasmic matrix itself or cell organelles as the rough endoplasmic reticulum, the nucleus or the perinuclear space may be inhabited (Smith and Arnott 1974). A third group of rod-like bacteria or spirochetes frequently lives on the outside of the flagellates being attached to the host cell plasma membrane by the cell tips or along their sides (Ball 1969, Bloodgood and Fitzharris 1976, Bloodgood et al. 1974, Hollande and Carruette-Valentin 1970, Kirby 1945, Smith and Arnott 1974, Tamm 1980). The respective body region covered by the epibiots may be typical of the flagellate species (Grassé 1938).

This study documents the diverse bacterial population of the hypermastigote *Joenia annectens* symbiotic in *Kalotermes flavicollis* and reports an unusual kind of bacterial granule formation and ingestion of the latter by the flagellate.

MATERIALS AND METHODS

Termites of the species *Kalotermes flavicollis* were received from the 'Bundesanstalt für Materialforschung' in Berlin where they have been in culture for several years. Members of the functional worker cast or nymphs were used.

The termite hindgut was pulled out and opened in a drop of 0.6% NaCl to obtain light microscopic preparations of the symbiotic flagellates. A drop of intestinal fluid expelled by the termites upon seizing them with a pair of tweezers could as well be used. To reduce damage by oxygen the samples had to be immediately covered with a cover glass.

For transmission electron microscopy (TEM) the intestinal fluid was directly submerged in the fixative, i.e., 2.5% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.2, for 30-60 min. The cells were further rinsed and postfixed with 1% osmium tetroxide for 30-60 min in the same buffer. Fixation was performed on ice or at room temperature. Embedding of rinsed flagellates in 1% agar facilitated their further handling. After dehydration in a graded series of ethanol they were embedded in Spurr resin or Epon 812. Thin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and observed with a Zeiss EM 10 electron microscope.

For scanning electron microscopy, fixation and dehydration was the same. Specimens were then transferred into absolute acetone and

pipetted into small caps covered by planktonic gauze prior to critical point drying with a Balzer's CPD 020. The dried flagellates were mounted on specimen stubs equipped with double-sided adhesive tape and coated with gold in a Balzer's SCD 040. The specimens were examined using a Philips SEM 515.

For microanalysis by electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) the flagellates were prepared as for TEM except that the fixative was free of OsO_4. Ultrathin sections which had to be extremely thin and unstained were observed with a Zeiss CEM 902 equipped with an integrated prism-mirror spectrometer. The inelastically scattered electrons with element-specific energy losses were used to obtain high resolution imaging of the nitrogen and phosphorus distribution of the sections (e.g., Ottensmeyer and Andrew 1980). Besides the element-specific ESI taken just above the edge of the electron absorption specific for N at 415 +/-10 eV (N_K = 402 eV) and for P at 150 +/-10 eV ($P_{L2,3}$ = 132 eV) a reference ESI below the edges (385 +/-10 eV for N, 115 +/-10 eV for P) was taken. The latter contains nonspecific background information.

RESULTS

Besides the large hypermastigote *Joenia annectens* a variety of smaller flagellates inhabited *K. flavicollis* (Fig. 1). *J. annectens* has been extensively examined on the light and electron microscopic level (Duboscq and Grassé 1928, Hollande 1979, Hollande and Valentin 1969, Lavette 1977). For completeness, the structural features of the animal were reviewed in short.

Numerous flagella were arranged in longitudinal rows forming a tuft on the, by definition, left anterior region of the elongated cell body (Figs. 2, 3). A single nucleus underlay that region. An axial rod, the axostyle, extended through the length of the body (Figs. 2, 4). It was composed of spirally wound microtubular lamellae. On the right side of the cell a fold of the axostyle, the capitulum, covered the nucleus (Fig. 4). The spherical particles which were especially frequent in the cytoplasm enclosed by the capitulum were hydrogenosomes. These are the anaerobic equivalents of mitochondria, fermenting pyruvate to acetate, CO_2 and H_2. Various parabasal elements emerged below the basal bodies. The cross-striated parabasal filaments were wound around the axostyle at the level of the nucleus. Here, they were

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Figs. 1-9. General morphology of *Joenia annectens*. 1 - The large hypermastigote *J. annectens* amidst different smaller flagellates in the gut fluid of *K. flavicollis*. Bar = 40 µm; 2 - *J. annectens* in phase contrast, depicting the tuft of flagella (F), ectobiotic bacteria (B), the nucleus (N) on top of the axial rod (= axostyle, A) and part of the Golgi apparatus (G). Bar = 20 µm; 3 - SEM of *Joenia* showing an apical ring free of adhering bacteria (arrow). Bar = 20 µm; 4 - The longitudinal section reveals a single nucleus (N) underlying the basal bodies of the flagellar tuft. Numerous dictyosomes (D) encircle the axostyle which is composed of spirally wound microtubular lamellae. A fold of the axostyle (A), the capitulum, encloses a cytoplasmic region rich in hydrogenosomes (H). Bar = 5 µm; 5 - Electron micrograph of rod-like bacteria attaching to the body surface. Bar = 2 µm; 6 - Spirochetes and cellulose in a food vacuole whose membrane is covered by attached bacteria. Differential interference contrast. Bar = 20 µm; 7 - An accumulation of non-attached, rod-like bacteria at the rear cell pole. Bar = 10 µm; 8 - Rod-like, attached bacterium in division. Bar = 0.25 µm; 9 - Rod-like bacteria partly resembling those in association with *Joenia* attach to the gut wall of the termite. Bar = 2 µm
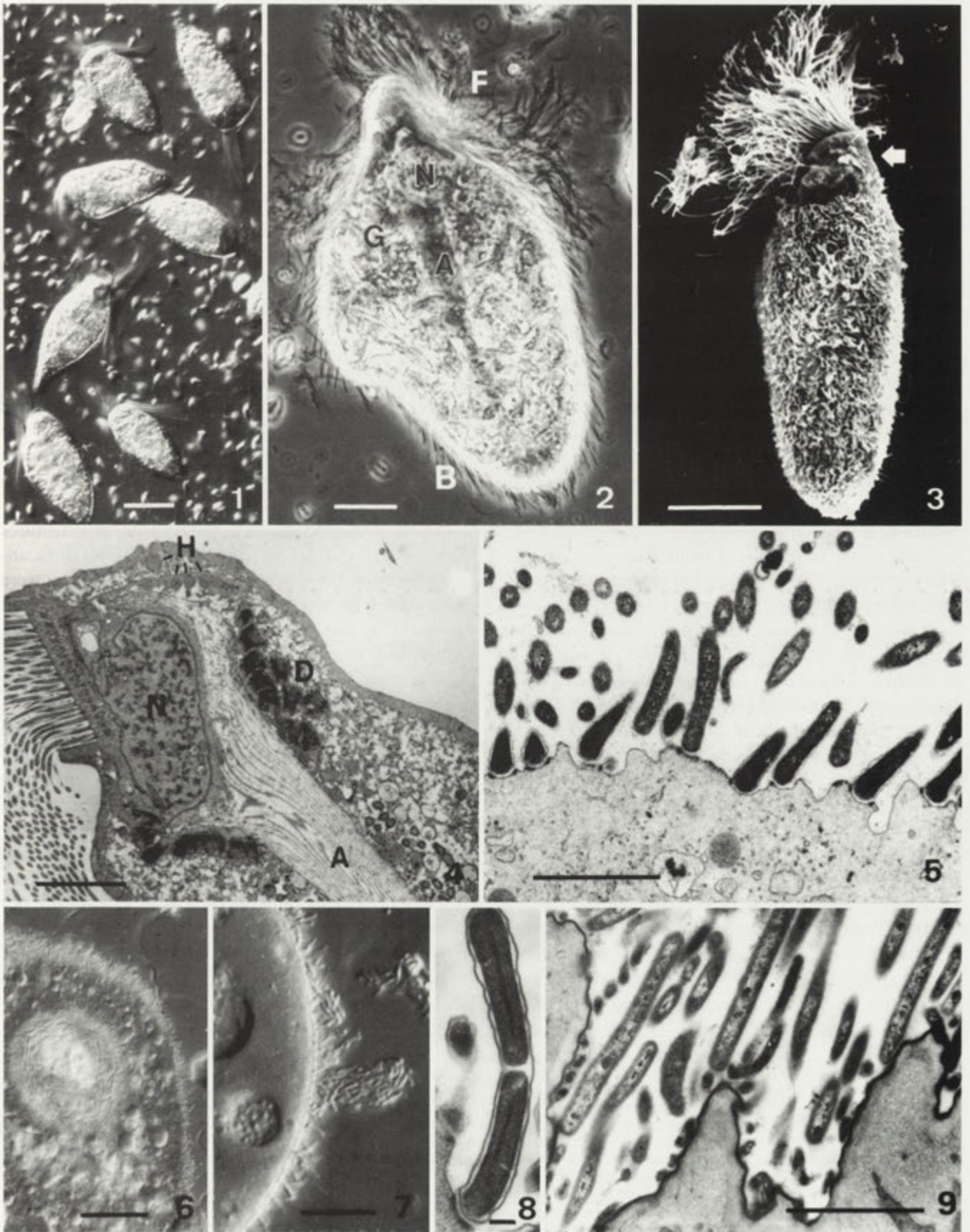
associated with numerous dictyosomes, which were thus called parabasal bodies (Figs. 2, 4).

1. Ectobiotic bacteria

The body surface of *J. annectens* was densely covered by rod-like bacteria (Figs. 2, 5) except for a ribbon-like region adjacent to the flagellar tuft (Fig. 3). The width of this zone varied from hardly being visible to extending over more than half of the body. Rarely, sporadic rod-like bacteria were found on the naked region indicating that this membrane area was basically capable of attaching bacterial ectobiots. Possibly, the vigorous beat of the flagellar tuft prevented a dense colonization. Small, cytoplasmic protrusions occasionally present in that region may have been due to fixation artifacts. Sometimes spirochetes adhered to the zone free of rod-like bacteria.

In contrast to the actively rotating spirochetes the rod-like bacteria were non-motile. Both types of attached microbes and bacteria freely living in the gut fluid were ingested by the flagellates in addition to wood particles. The membrane of the newly formed food vacuoles was thus covered by the bacteria on its internal side (Fig. 6).

It was in question whether the rod-like bacteria only occur attached to a flagellate or whether they are as well able to inhabit other ecological niches. Occasionally, accumulations of unattached bacteria were found in the gut fluid being identical in fine structure to the slender and thick rods normally colonizing the surface of the flagellate. As well, bacterial aggregations which on the light microscopical level seemed to be identical to the attached types loosely adhered to the posterior cell pole of *Joenia* (Fig. 7). These might be daughter individuals released into the medium by transverse fission (Fig. 8).

Also, bacteria were found adhering to the intestinal wall of the termite. The cuticular intima overlaying the epithelial cells was densely covered by various types of bacteria (Fig. 9). They were attached to the intima by the their tips or sides. Some of them closely resembled the ectobiotic rods residing on the flagellate surface, implicating that the same species may have attached either to the flagellate membrane or to the hindgut wall.

1.1. Rod-like bacteria

Morphology of the bacteria

Only one type of rod-like bacteria adhering to the surface of *J. annectens* was described by Hollande and Valentin (1969). We found three different morphological forms: a very long slender type, a short slender type, and a short thick type. Generally, one kind of bacterium strongly prevailed on a host flagellate. Clearly mixed populations, however, occurred as well (Figs. 3, 10, 11), indicating that there did not exist different flagellate strains possessing an own bacterial ectobiont. In the case of mixed bacterial populations the different microbes were not uniformly distributed over the surface but were aggregated together in patches (Fig. 11).

For the characterization of the bacterial type-features light microscopical (LM), transmission (TEM) and scanning electron microscopical (SEM) techniques were combined. The long, slender form extended over about 10 µm (LM) and measured 200 nm (SEM) in diameter. A medium length of 3.3 µm (LM) combined with a medium width of 230 nm (SEM) was typical of the short slender bacteria. 270 nm were measured as the diameter of the thin bacteria if the width was measured on ultrathin sections, where the short and long types could not be distinguished. This deviation in width may have been explained by swelling phenomena which were reflected in a wavy, loosely fitting outer membrane layer of the typical gram-negative cell boundary (Fig. 12). A murein-layer between the two membranes was not detectable. Pure differences in length were not sufficient to separate species. Bacteria may possess polymorphic developmental cycles with a succession of filamentous types, long and short rods and cocci (Weide and Aurich 1979). The third bacterial type had ap-

Figs. 10-18. Ectobiotic bacteria on *J. annectens*. 10 - SEM of the rear cell pole showing long and short rod-like bacteria being attached to the same flagellate. Bar = 10 µm; 11 - SEM of attached bacteria. Thin and thick types with their adhering poles broadened club-like. Fluffy glycocalyx material connects the bacteria with the flagellate (arrow). Bar = 1 µm; 12 - Prokaryotic-eukaryotic cell junction. In addition to the plasma membrane one further membrane covers this thin, gram-negative bacterium. Glycocalyx material spans the regular gap between bacterium and the indented host cell membrane (arrows). The flagellate membrane is supported by electron dense material at the borders of the attachment site (arrowheads). Bar = 0.1 µm; 13 - Thin and thick bacteria on the same flagellate. The regions of the attachment sites may protrude. The contact zones are completely underlain by electron dense material. Bar = 0.5 µm; 14 - Electron dense underlayer of an attachment site turned inward at its borders (arrows). Bar = 0.1 µm; 15 - Protruding attachment site free of an associated bacterium. Bar = 0.1 µm; 16 - Two types of spirochetes adhering to *Joenia*. One is slightly curved and possesses a tube-like appendage (arrow). The other type is straight and has a wavy sheath (arrowhead). Bar = 1 µm; 17 - Spirochete with the tube-like appendage adhering to the flagellate. Bar = 0.1 µm; 18 - Second spirochete type. An electron dense plate supports the non-deformed membrane of the flagellate. Bar = 0.1 µm

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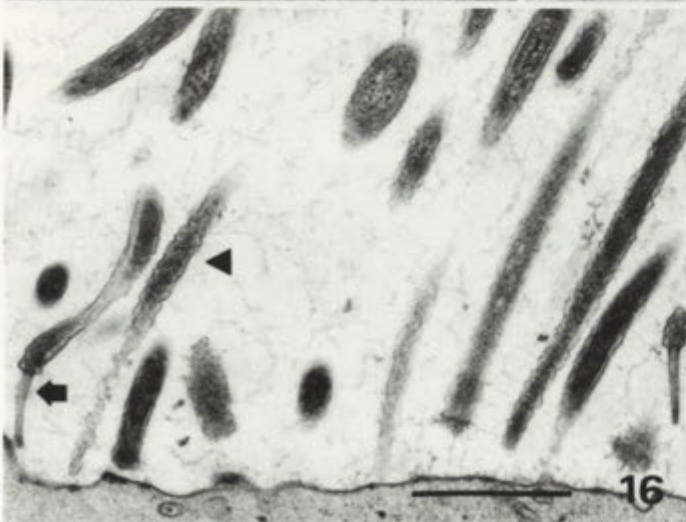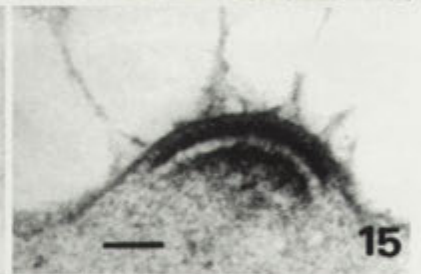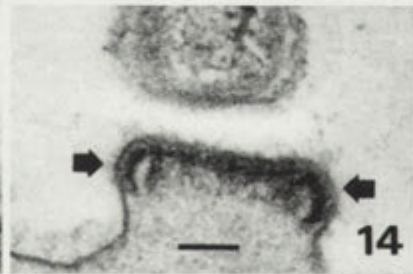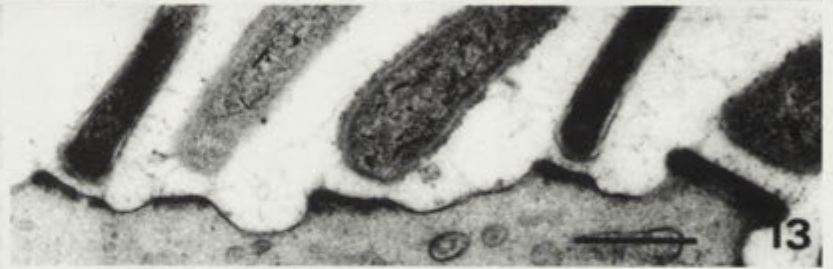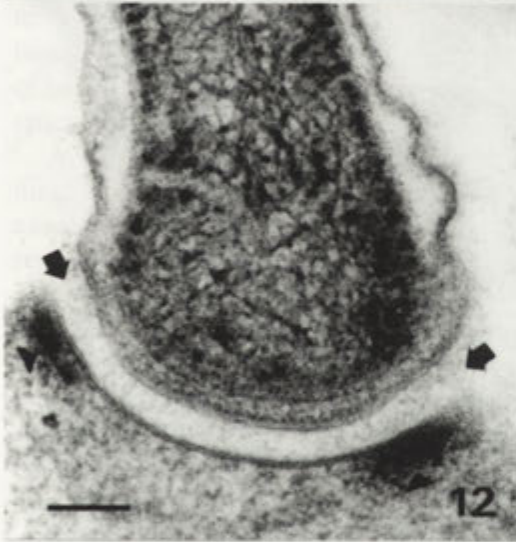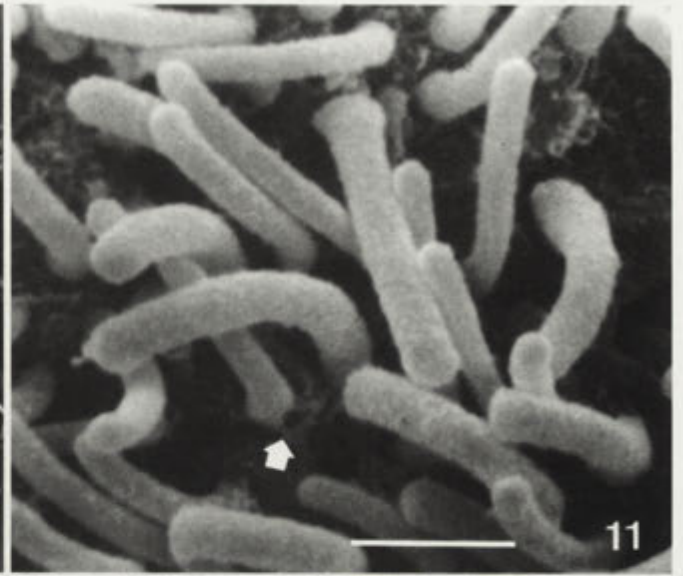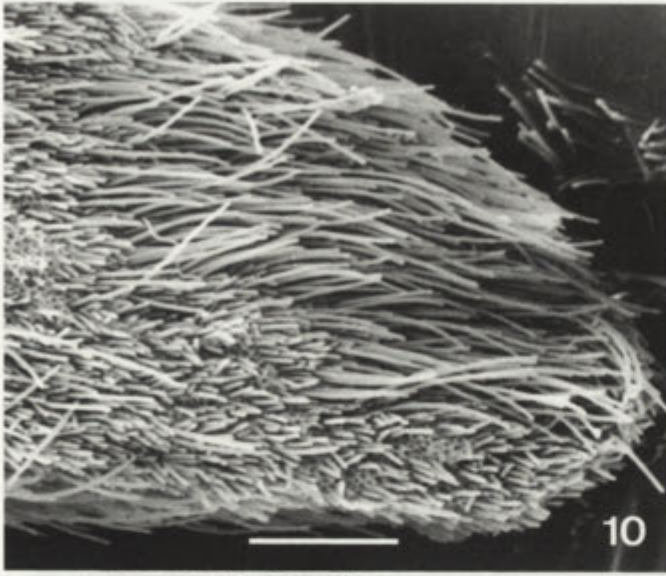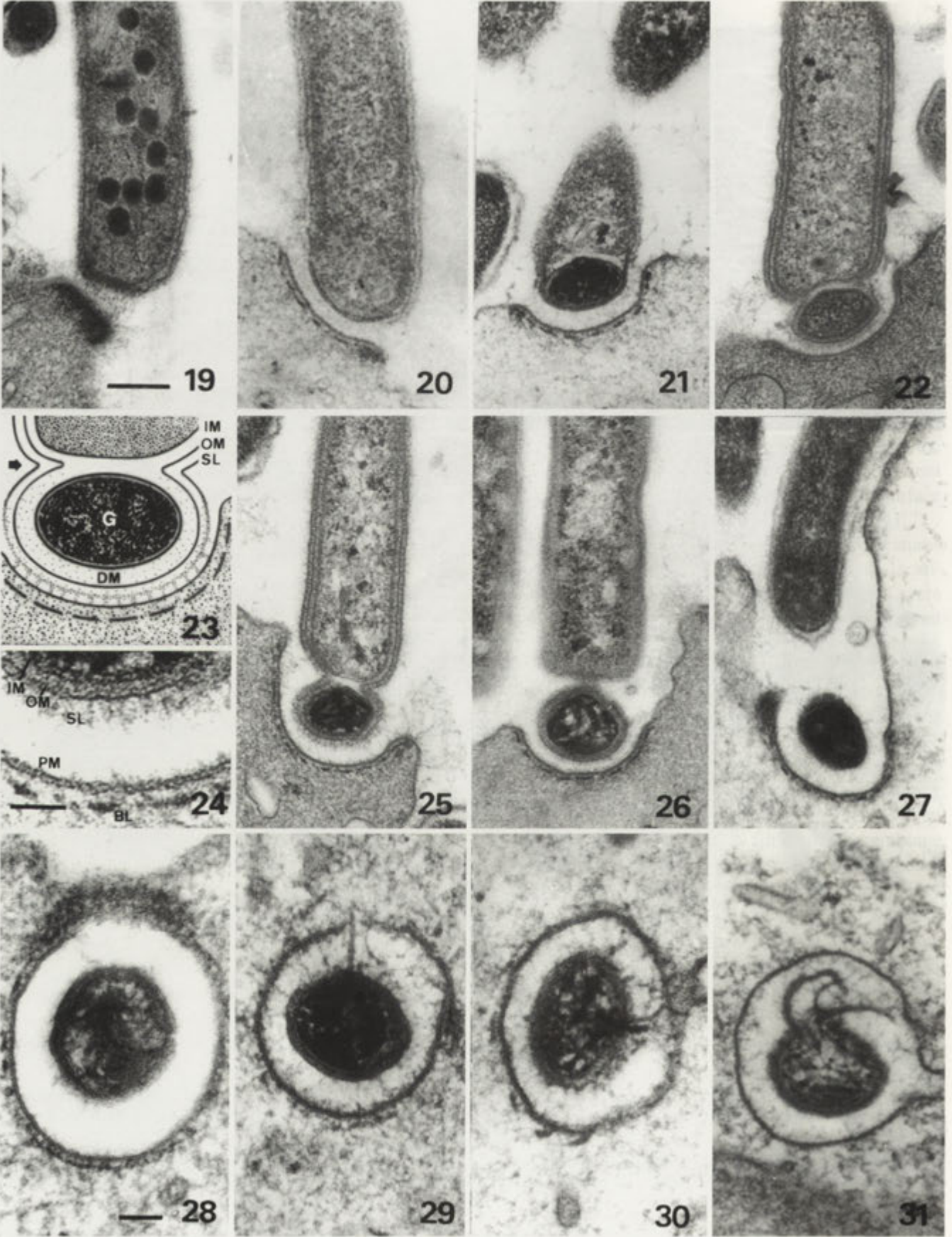
proximately the same length as the slender short type (3 µm in general, maximally 6 µm) but was somewhat thicker, measuring 350 nm (REM, TEM). Its cell wall seemed to be more compact (Fig. 22). The outer membrane did not lift off but remained in a constant distance of about 12 nm to the inner cytoplasmic membrane. An additional, slightly diffuse layer of about membrane thickness covered the thick bacteria.

Structure of the attachment sites

The point where the bacteria were attached to the flagellate was highly specialized. The attaching pole of the bacteria was broadened club-like (Fig. 12). All cell wall components were continuous at the adhering tips. The additional outer layer of the short, thick rods seemed to disintegrate to a certain degree into a more diffuse layer at the attachment site (Fig. 20). Glycocalyx material of bacterial and/or flagellate origin spanned the regularly spaced gap between the two organisms (Fig. 12).

At the prokaryotic-eukaryotic cell junction the membrane of the flagellate was indented, copying the exact shape of the bacterial cell pole. The complete region of attachment might project so that the bacteria were situated on small elevations of the flagellate body. Electron dense material always underlay the flagellate's membrane at the contact zone. The specific pattern of material arrangement could, however, vary. At least the border of the adhesion site was supported by the material (Figs. 5, 12). Additionally, the total area of the cell junction was sometimes equipped with an amorph, electron dense layer (Fig. 13). The borders of this layer sometimes were curved inward (Fig. 14, arrows).

In spite of the specialized morphology of the adhesion site, the ability to attach to the membrane of

Joenia was not restricted to one bacterial cell pole. When an other region of the bacterial surface came into contact with the flagellate surface a similar kind of cellular connection could be formed (Fig. 27). Thus the cell tip of the microbes did not seem to possess exclusive properties enabling attachment.

From time to time spots with the characteristic fine structure of attachment sites were found which were depleted of associated bacteria (Fig. 15). The surface of these spots was not indented to fit a bacterial cell pole but was convexly arched. The electron dense layer was clearly visible and glycocalyx material normally being associated with a bacterium was obvious. Such free attachment sites may have been due to artificial distortion of the ectobiots during electron microscopic preparation or they represented pre-formed regions to catch and attach free-living bacteria.

1.2. Spirochetes

Besides the rod-like bacteria motile spirochetes of about 20 µm in length attached to *Joenia* (Fig. 16). They were not found with the same regularity as the rod-like bacteria. None could be present just as well as great numbers, even hindering the normal movement of the flagellate. They found their attachment sites inbetween the ectobiotic rods. Two different types occurred. The first was slightly curved and possessed an elongated tube-like appendage (500 nm long and 70-80 nm wide at its basis; Fig. 17). The cytoplasmic cylinder elongated into that 'tube'. Alongside the 'tube' the cellular sheath was closely fitting in contrast to the rest of the cell body where it lifted off from the cytoplasmic cylinder in slight waves. The appendage made contact to the flagellate mem-

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Figs. 19-31. Granule formation in adhering rod-like bacteria. Figs. 19-22 and 25-27 bar = 0.2 µm, Fig. 24 bar = 50 nm, Figs. 28-31 bar = 0.1 µm. Except Fig. 27 which depicts a thin bacterial type all figures show thick, short bacteria. 19 - Dark particles occurring in an attached bacterium are rather viruses than developmental stages of granule formation; 20 - Possible early stage of granule formation. A cytoplasmic area seems to be demarcated at the adhering cell pole and the prokaryotic-eukaryotic cell junction is delineated by a thin, broken, electron dense layer, typical of granule-bearing bacteria; 21 - An electron dense granule was formed which fits the width of the bacterium; 22 - The bacterial cell borders invaginate adjacent to the granule; 23 - A scheme elucidates the course of the wall structures of this stage. The bacterium's outer membrane (OM) and the additional surface layer (SL) which are continuous around the granule (G) invaginate (arrow). The surface layer loses its clear shape and gets diffuse at the attachment site. The inner, cytoplasmic membrane (IM) separates the bacterial cytoplasm from the granule which has an own delineating membrane. Dense material (DM) fills the gap between the inner and the outer membrane alongside the granule; 24 - Wall structures of a granule-containing, attaching bacterium. BL = fine, broken line supporting the flagellate plasma membrane (PM) at the attachment site, further abbreviations as in Fig. 23. Bar = 50 nm; 25 - Deep invagination of the bacterial wall structures adjacent to the granule; 26 - The granule is pinched off by fusion of the invaginating membranes; 27 - An attachment site largely surrounds a granule lying close to a bacterium. Along one side of the bacterium a contact zone is formed with the flagellate surface. 28 - Granule being enclosed in an endocytotic vesicle. The broken line typical of attachment sites involving granule-bearing bacteria supports the membrane of the vesicle; 29 and 30 - Pin-like outgrowths of the granules being into contact with the membrane of the vacuole. Glycocalyx material spans the space between granule and vacuole membrane; 31 - Beside the rise of outgrowths the shape of the vacuoles becomes irregular and the membrane is no longer supported by electron dense material
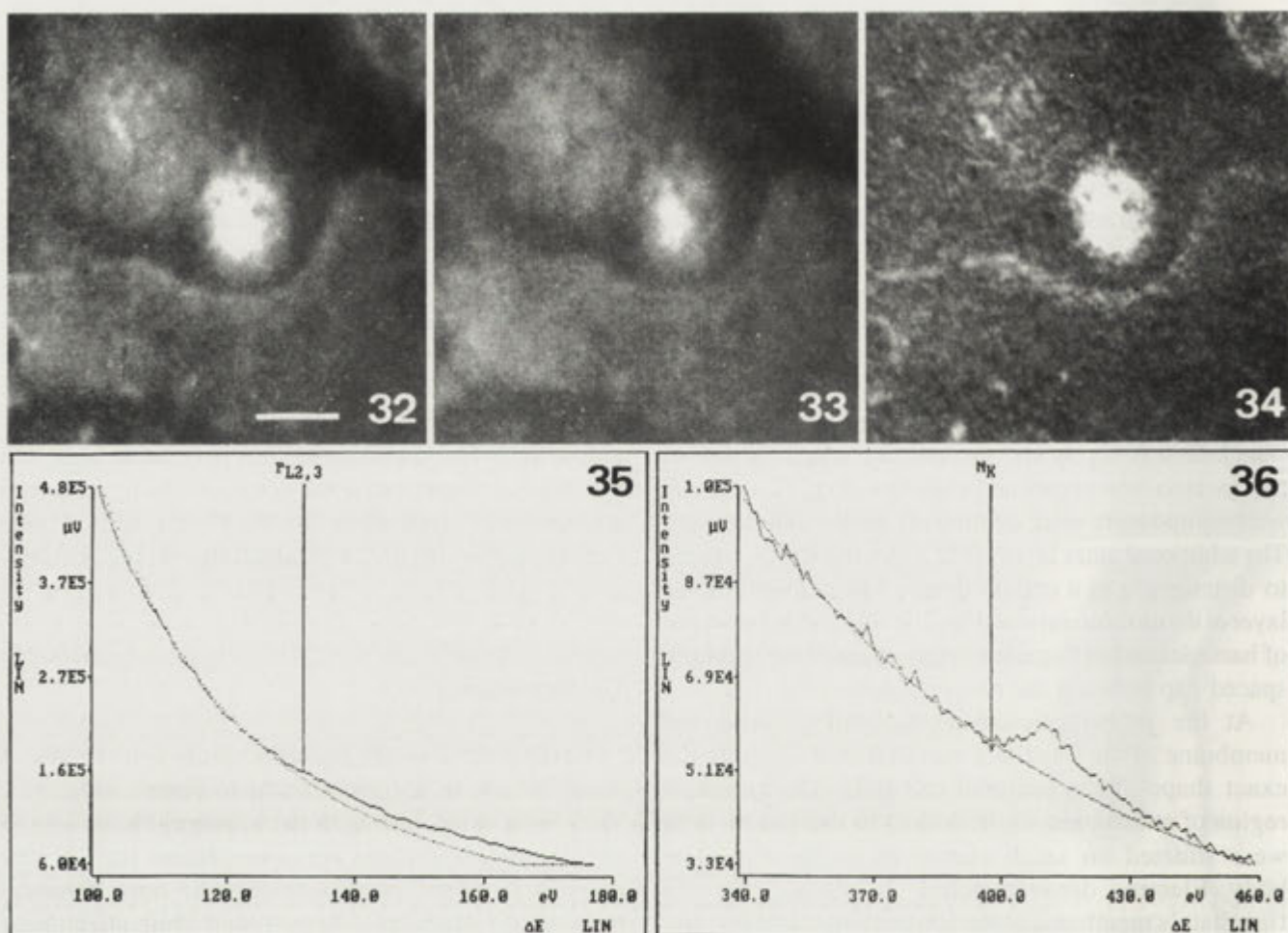
Figs. 32-36. High-resolution microanalysis of bacterial granules. 32 - Electron spectroscopic image (ESI) taken just above the phosphorus absorption edge ($P_{L2,3}$ = 132 eV). Bar = 0.1 μm; 33 - ESI of the same area taken at 115 +/- 10 eV as reference below the phosphorus absorption edge. The differences betweeen the images in Figs. 32 and 33 should be due to phosphorus alone; 34 - Photographic 'subtraction' of Figs. 32 and 33. The net phosphorus distribution is shown by white areas in the image; 35 - Electron energy loss spectrum (EELS) of a granule depicting the presence of phosphorus ($P_{L2,3}$ edge at 132 eV). The dash line represents the extrapolated background; 36 - EELS depicting the presence of nitrogen (N_K egde at 402 eV)

brane. Little electron dense material seemed to underlie the host cell membrane at this point.

The second type was a slender (0.18 μm wide) and straight form ultrastructurally not much reminding of a spirochete (Fig. 18). Its outer sheath was remarkably wavy. A layer of electron dense material supported the flagellate membrane at the attachment site. Glycocalyx material spanned the gap between bacterium and protozoon. The surface of *Joenia* was plain at the cell junctions formed by either of the two types of spirochetes.

Free in the intestinal fluid there were at least two other types of large spirochetes belonging to the family Pillotaceae (*Pillotina* spec. and *Hollandina* spec.; Bermudes et al. 1988, To et al. 1978) which were not dealt with in the scope of this study.

1.3. Granule forming rod-like bacteria

Both, thick and thin types of rod-like bacteria could possess an unusual granule at their adhering cell poles. Different stages of granule formation and pinching off the granule seemed to be present. In no other bacterial cell region could such large granules be recorded. Parallel, the morphology of the prokaryotic-eukaryotic cell junction changed.

In search for stages leading to granule formation bacteria with one or several small (average 80 x 115 nm), dark bodies were found, which, however, rather seemed to be virus particles (Fig. 19).

Other changes were depicted in Fig. 20. The amorph layer of electron dense material underlying the attachment site, here, was a distinct line which was often

interrupted. This fine and frequently broken line was typical of granule-bearing prokaryotic-eukaryotic cell junctions (Figs. 21-26). Furthermore, at the attaching cell pole an oval cytoplasmic zone not much differing in fine structure from the rest of the bacterial cytoplasm seemed to be demarcated.

A considerable quantity of bacteria included a large granule at their attaching cell pole which was conspicuous because of its unusually high electron-density (Figs. 21, 25-31). Except for some small lighter and unregularly shaped spots no internal structures could be detected. Sometimes, the border of this dark inclusion body appeared more electron lucent (Fig. 21). Its shape was oval to spherical, fitting into the normal cell diameter. The size was relatively uniform (about 240 nm in width and 170 nm in height). The outer bacterial wall structures consisted of an outer membrane and an additional layer with regard to the small, thick bacteria. All these structures were continuous around the granule (Figs. 21-25). However, the inner, cytoplasmic membrane separated the bacterial cytoplasm from the granule which had an own delimiting membrane (Figs. 21-25). In regions where the bacterial wall layers covered the inclusion body, dense material filled the gap between the outer membrane and the inner one (Fig. 24). The inner membrane of the granule could be easily recognized in periodic acid bleached sections (30 min 5% periodic acid; Fig. 22). The center of the inclusion body appeared lighter and granular in this bleached specimens but did not reveal further structures.

Some bacteria visibly narrowed in the region adjacent to the granule. The outer wall layers were circularly invaginated into the gap between the granule and the bacterial plasma membrane (Figs. 22, 25). For better understanding, a scheme depicts the diverse wall structures showing a thick bacterium (Fig. 23). The bacterial wall layers invaginated very deeply and seemed to fuse when they met (Fig. 26). Furthermore, granules lying close to the cell pole of a bacterium and adhering to a typical attachment site were found (Fig. 27).

Attachment sites of granule-bearing bacteria were deeper invaginated into the flagellate's cytoplasm than those of normal bacteria (Figs. 21-26). Especially granules which lost their contact to the bacterium were deeply enclosed by the borders of the attachment site (Fig. 27). Others were completely surrounded by the attachment site-like plasma membrane region thus taking up the granule into the cytoplasm (Figs. 28-30). This endocytotic vesicles could lie directly beneath the plasma membrane of the flagellate (Fig. 28) or deeper in the cytoplasm (Figs. 29-30). Most of this vesicles were allround equipped with the typical features of an attachment site, i.e., a thin, broken line supporting the membrane and glycocalyx material connecting the granule with the membrane (Figs. 28-30).

Some curious outgrowths were visible on the surface of granules which lay deeper in the cytoplasm. They were pin-like, often curved and seemed to make contact with the surrounding membrane (Figs. 29-31). The supporting fragments of the thin, electron dense layer were less prominent or even totally disappeared (Fig. 31). The missing characteristics of former attachment sites and the increasing deformation of the granules made it impossible to further follow the fate of the inclusion bodies.

To gain information about the nature of the granules, electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) were applied. Heavy elements as for example iron could not be found but instead phosphorus and nitrogen signals were distinct. The P distribution of a granule-bearing bacterium was depicted in Fig. 32 by ESI. Bright white regions corresponded to the zones of high P content. A comparison with the background level taken below the characteristic edge (Fig. 33) gave an impression of the net content of P (Fig. 34). The element specific curves gained by EELS supported a strong enrichment of P (Fig. 35) and N (Fig. 36) within the granules.

2. Endocytobiotic bacteria

2.1. Bacteria in the nucleus

Two types of bacteria-like inclusions regularly occurred within the nucleus and did not seem to cause damage (Fig. 37). One form was coccoid with a width of about 500 nm. An electron lucent zone separated the plasma membrane from a smooth, (18 nm) thick wall layer. The cytoplasm included large light zones, probably reserve material. Division stages were frequent. The second type was spherical as well but measured less in width (300 nm). Its outer cell boundary was thinner and conspicuously wavy. No inclusions were visible in its dense cytoplasm.

2.2. Bacteria in the cytoplasm

On the light microscopical level an accumulation of granules and rods was visible around the axostyle. In ultrathin sections these inclusions could be recognized
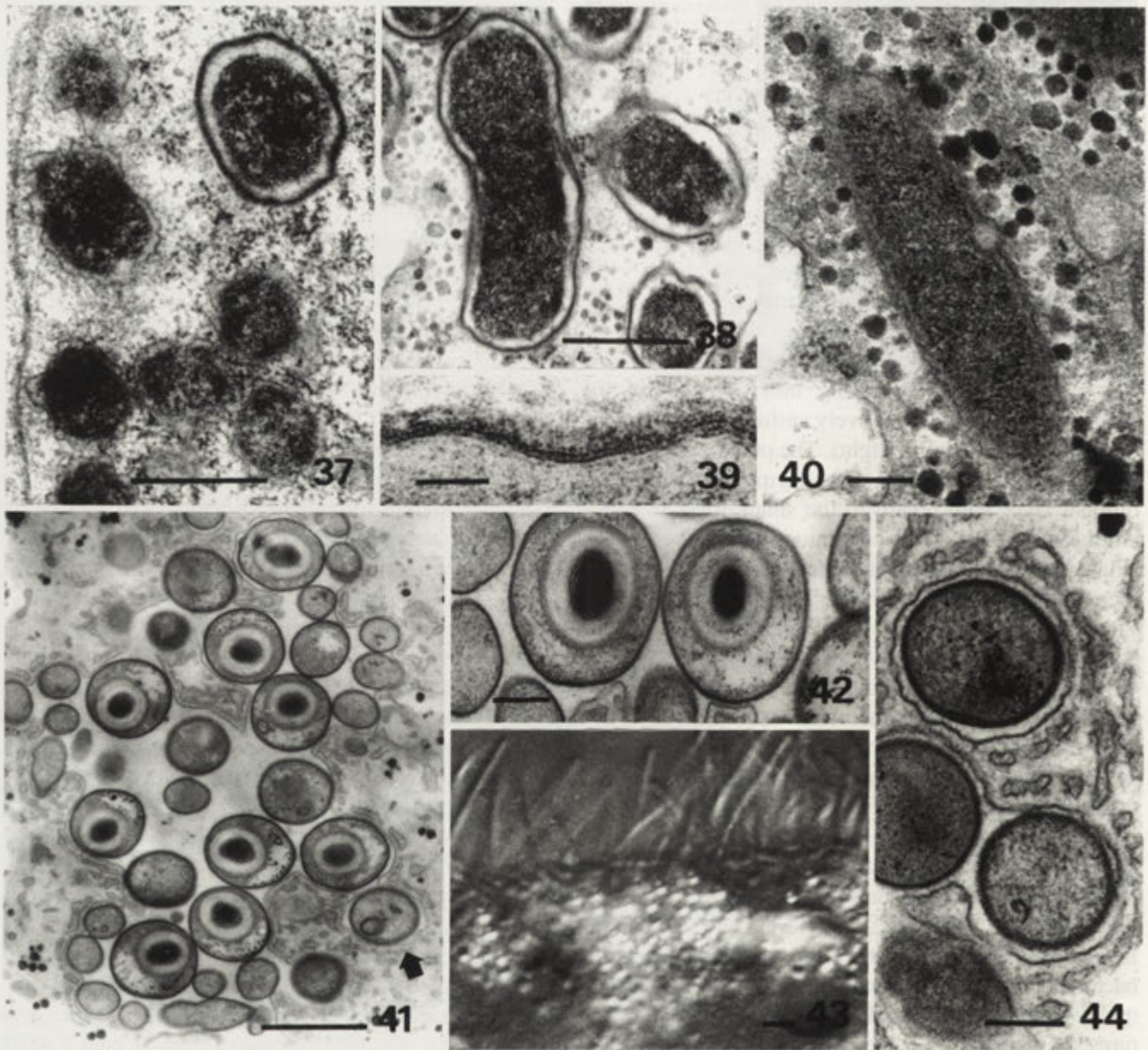
Figs. 37-44. Endocytobiotic bacteria. 37 - Two types of coccoid bacterial inclusions occur within the nucleus. One form has a thick smooth border whereas the second, smaller type is surrounded by a thin, wavy line. Bar = 0.5 μm; 38 - Irregularly shaped, rod-like bacterium within the cytoplasm. A clear space separates the dense, central cytoplasm from the dark cell border. Bar = 0.5 μm; 39 - The cell border of the irregularly shaped bacterium consists of a membrane equipped with fluffy material on the outside. Bar = 50 nm; 40 - Cytoplasmic rod-like bacterium which is typically surrounded by glycogen granules. The outer membrane is slightly wavy. Bar = 0.2 μm; 41 - Vacuole with spore-forming bacteria. Different developmental stages. Single bacteria are pinched off from the vacuole (arrow). Bar = 1 μm; 42 - Spore-containing bacteria possess a dark inclusion body surrounded by several layers, i.e, the spore. Bar = 0.2 μm; 43 - Interference contrast picture showing an accumulation of refractile granules probably identical to the spores. Bar = 2 μm; 44 - The membrane of the bacteria-containing vacuole is supported by vesicles. Bar = 0.2 μm

as various types of bacteria or as hydrogenosomes. Some kinds of bacteria were digested in small vacuoles, others looked normal and seemed to live here as endocytobiots. The spectrum and the abundance of the bacteria being present could vary in different specimens.

2.2.1. Irregularly shaped bacteria

Rod-like bacteria with an irregular shape lay in direct contact to the cytoplasm (Fig. 38). They measured 380-530 nm in width.

The longest forms had a length of 2.4 μm. A gap often separated the dense central cytoplasm from the thick, dark cell boundary, which consisted of a membrane equipped with fluffy material on its outside (Fig. 39). The features of the cell boundary seemed to correspond to gram positive wall structures.

2.2.2. Rod-like bacteria surrounded by glycogen granules

There is a second type of rods which was not enclosed in a vacuole (300 nm in width, maximally measured length 2 μm; Fig. 40). Accumulations of glycogen granules surrounded these bacteria excluding other cytoplasmic structures. Glycogen granules were scarce in the cytoplasm between the bacteria. The cell boundary of the bacteria was slightly wavy but did not lift off. The cytoplasm was finely granular.

2.2.3. Bacteria enclosed in vacuoles

The flagellates engulfed various kinds of intestinal bacteria and spirochetes in order to digest them. These phagocytized forms did not contribute to the endocytobiotic population. It was interesting, nevertheless, that, e.g., an engulfed bean-like, dark bacterium could attach to the membrane of the food vacuole by fluffy glycocalyx material (not shown). This type of bacterium was never found attached to the surface of *Joenia*.

Other vacuoles exclusively contained one type of spore-forming bacteria (Fig. 41). The normal, spore-less bacteria were about 600 nm long and 280 nm wide, possessing tapered cell poles. Their cell boundary was simple, the cytoplasm spongy. Spore-containing cells were of double thickness. Spore-formation was not followed in detail. The spores were slightly elongated and positioned acentrically within the cell (Figs. 41, 42). The spore-containing vacuoles were visible already in the light microscope as clear defined regions filled with refractile granules (Fig. 43).

The vacuole enclosing the bacteria was not bounded by a simple membrane but additionally equipped with a layer of flat vesicles supporting the vacuolar membrane (Fig. 44).

DISCUSSION

A variety of bacteria inhabiting the surface, the cytoplasm or the nucleus of the termite flagellate *Joenia*

annectens have been described in the present paper. In no case do these co-inhabitants seem to damage their host. Bacteria-like organisms inhabiting, e. g., the nucleus of protozoa are by no means exceptional (Ball 1969, Kirby 1944, 1964, Lee et al. 1985). In some cases a parasitic way of life seems to be apparent. A drop in fission rate and fission anomalies may for example result from an infection of *Paramecium* nuclei with *Holospora* species (Görtz 1988). The various endocytobiotic bacteria in the cytoplasm possibly live in a symbiotic relationship with their host. Bacteria are mostly called 'symbiots' if their association with the protists is not obviously parasitic. For a more differentiated definition and discussion of the possible roles endosymbiots may play in protozoa see Heckmann (1977).

Certain anaerobic protozoa as for example free-living or rumen ciliates are associated with methanogenic bacteria (Fenchel and Finlay 1991, Wagener et al. 1990). These methanogens use the hydrogen being released by hydrogenosomes to reduce carbon dioxide to methane. Under utraviolet irradiation methanogenic bacteria fluoresce due to their characteristic coenzyme F_{420} (Doddema and Vogels 1978). Using this tool none of the bacteria associated with *J. annectens* could here be recognized as a methanogen. Other functions of the endocytobiotic bacteria of termite flagellates could be fixation of nitrogen (Smith and Arnott 1974) or production of enzymes for cellulose digestion which has been a matter of discussion for a long time (Honigberg 1970, Hungate 1955, Kirby 1941a). Pointing against a role in cellulose digestion is the fact that axenically cultured *Trichonympha* harbored no endocytobiotic bacteria and could nonetheless survive and metabolize cellulose (Yamin 1981).

In addition to endocytobiotic bacteria, ectobiotic ones covering the body surface of protozoa are a common phenomenon. The attached species may be motile spirochetes as those of *Mixotricha paradoxa* (Cleveland and Cleveland 1966) or motile rods as those of a devescovinid termite flagellate (Tamm 1982). In these cases, the synchronously moving microbes obviously serve to propel their host cell. Most of the described ectobiots, however, are non-motile or do not move coordinately due to an irregular attachment pattern (Beams et al. 1960, Bloodgood and Fitzharris 1976, Kirby 1941b, 1945). Their advantages for the flagellate may lie on the nutritional sector. Other ectobiots as those of the ciliate *Euplotidium itoi* contain a complex extrusive apparatus and thus resemble extrusomes in some respects (Verni and Rosati 1990).

Concerning *J. annectens*, rods which might have risen from cross-fission of attached bacteria are frequently seen to form a loose accumulation adhering to the posterior cell pole. Bacteria of similar fine structure were found free in the intestinal fluid. Maybe, the daughter cells could not find any space for attachment since the surface of *Joenia* is closely covered with bacteria. Among the various bacteria adhering to the cuticular wall of the paunch the same type of rods was found. After successive divisions in the paunch of molting nymphs, *J. annectens* gets rid of its ectobiots (Lavette 1969a). All these observations might lead to the conclusion that these bacteria are not essential symbiots of *Joenia*.

Possibly, the ectobiots are grown to get phagocytized as food, supplementing, e.g., the nitrogen-poor wood diet of the lower termite flagellates (Lavette 1969b). The attached rod-like bacteria of *Joenia* frequently get enclosed in food vacuoles and are digested. The same was reported for the ectobiots of other protozoa such as the marine ciliates *Kentrophoros* and *Parablepharisma* (Fenchel and Finlay 1989) or a flagellate inhabiting the cockchafer (Brugerolle 1981). The microbes, as well, may benefit from their host. In our case the danger for the small bacteria to be expelled with the excreted gut content is lowered by attachment.

Regardless of the advantages and disadvantages of the prokaryotic-eukaryotic association the question is posed how the organisms manage to get into contact. In all cases special junctional zones are developed by the protozoon and/or the ectobiotic bacterium. Only endocytobiots do not possess any specialized structure of attachment. Keeping to the ectobiots a common feature of their junctions is a more or less structured electron dense material supporting the region of contact. Often filamentous extracellular material bridges the space between the two contacting partners. It is not known from which partner/s it originates (Tamm 1980). The prokaryotic-eukaryotic contact zones morphologically closely resemble adhesive junctions such as (hemi)desmosomes and as the latter they provide a strong mechanical anchorage since bacteria persist even on disrupted fragments of the host membrane (Tamm 1980).

The attachment sites on termite flagellates may be located in special indentations or on elevations, uniformly distributed or unregularly scattered over the body surface (Bloodgood and Fitzharris 1976, Cleveland and Grimstone 1964, Grassé 1938, Tamm 1980). Not only the protozoon develops such specific sites but also the

bacteria may possess structures assigned for attachment as, e.g, tube-like or more complexly structured appendages of spirochetes (Bloodgood and Fitzharris 1976, Smith and Arnott 1974). In our study a long, slender microbe adhering to *Joenia* with such a tube-like appendage was described. Basing on the rotating mode of movement and the close ultrastructural similarity to spirochetes being described by other authors (Bloodgood and Fitzharris 1976, Holt 1978, Smith and Arnott, 1974) we concluded that it was a spirochete although axial fibrils could not be demonstrated. The same is valid for the second spirochete-like bacterium attaching to *Joenia* without special structures. Single axial fibrils are hard to detect in ultrathin sections. Shadowed replicas were the only means to demonstrate the axial filament of spirochetes attaching to *Pyrsonympha vertens* (Smith and Arnott 1974).

It was suggested that only the partner benefiting from the prokaryote-eukaryote association will tend to form a structural specialization at the attachment site (Bloodgood and Fitzharris 1976). Some reports described ultrastructural differentiations in both the bacterial and the host cell membranes of gut flagellates (Cleveland and Grimstone 1964, Tamm 1980) or intestinal epithelial cells (Neutra 1980, Wagner and Barrnett 1974). A mutual benefit for the partners of such a kind of association is inferred in many cases (Cleveland and Grimstone 1964, Hollande and Valentin 1969, Tamm 1982).

The number of attachment sites is not static. Spirochetes, e.g., may detach from the flagellate surface at random (Holt 1978). Detachment of the microbes and attachment sites is postulated to occur for example by formation of vesicles just internal to the attachment site (Holt 1978, Smith et al. 1975). A possible developmental sequence for the formation of new cell junctions was proposed (Smith et al. 1975). Early attachment sites seemed to be formed independent of contact to a spirochete. Other flagellates (*Barbulanympha, Urinympha*) contacting rod-like bacteria never showed attachment sites without adhering bacteria (Bloodgood and Fitzharris 1976). Concerning *J. annectens* there is a permanent need for new contact zones since adhering bacteria get ingested including their cell junctions and since daughter individuals risen by cross-fission have to form new attachment sites. The ectobiots of the marine ciliate *Kentrophoros* (Fenchel and Finlay 1989) and for example long rods attaching to the chitinous gut wall of cockroaches (Foglesong et al. 1975) divide by longitudinal fission which facilitates adhesion of the

daughter individual. Cross-dividing bacteria, e.g. spirochetes, may bend so that the posterior specimen can secure itself before division is completed to a nearby vacant attachment site to which it is attracted by chemotactic substances (Breznak 1973). On *J. annectens* attachment sites free of bacteria are only rarely found and seem to be artifactually deformed, implicating that the junctional structures are not formed in expectation of a bacterium settling down but are induced when a bacterium comes into contact with the surface. Different fine structures of the prokaryotic-eukaryotic contact zones, i.e, electron dense material supporting only the borders or the total contact zone, may represent different stages of junction formation. Another fact supporting the idea of junction induction is that the rod-like bacteria which normally attach by their tips can also form elongated cell junctions at their sides if they come near to the flagellate's surface. The composition of the bacterial surface thus seems either to be similar at the tips or sides of the cell or its properties may adapt to the particular situation. Normally, however, bacteria of one type show attachement to a special host species at only one of the mentioned bacterial cell regions. How is this managed if the potential for adhesion is distributed all over the surface?

Another question which arises is whether the host cell is able to discriminate between different bacteria coming into contact with its surface. A great number of microbial species inhabit the gut but only a restricted number of rod-like forms or spirochetes may attach. The nature of such interactions on the molecular level is poorly understood. Several mechanisms may be involved. Firstly, attachment may be mediated by specific recognition factors. For example, bacterial surface proteins (comparable to plant lectins) may interact with specific glycosylated components of the host cell surface (Gibbons 1980, Ofek et al. 1977). Obviously, glycocalyx material spans the gap between *Joenia* and its ectobiots. The adhesion-promoting role of bacterial capsules and proteinaceous pili is discussed controversely (Rosenberg and Kjelleberg 1986). In many cases, adhesion may be evoked by hydrophobic interactions (Rosenberg and Kjelleberg 1986). Many researchers agree that hydrophobic interactions mediating bacterial adhesion are not dependent on molecular complementarity, i.e., are nonspecific. Concerning adhesion to host cells, however, the possibility has been raised that specific recognition mechanisms between hydrophobic moieties and stereospecific receptors do occur (Abraham et al. 1983, Ofek et al. 1983).

A conspicuous feature of the rod-like bacteria adhering to *Joenia* was the formation of a granule. Diverse morphological situations were found which implicate the passing through of a special process. In short, the granules seemed to be pinched off from the bacteria and to be further engulfed by the flagellate.

We wondered about the nature und the purpose of the granules. They differ from the bacterial inclusion bodies which have been described up to now. The high electron density and the position at the cell pole could lead to the assumption that they might contain polyphosphate which is a typical reserve material of bacteria. However, the granules never evaporated under the electron microscopical beam nor did they tend to get lost during ultrathin sectioning, features being typical of polyphosphate granules (Jensen 1968). In fact, the presence of P, and additionally of N was proven by ESI and EELS, supporting the reserve material identity. The complex wall pattern of the granule, however, contradicts this theory. Bacterial inclusions may at the most be surrounded by a simple proteinaceous membrane of 2-4 nm thickness dissimilar to the unit membrane (Weide and Aurich 1979). Regardless of the chemical composition, in our case exchange of material might be the possible purpose of the described phenomenon. The content of the granule may be waste for the bacterium but nourishment for the flagellate.

Another idea is that the granule might be an unusual kind of spore. Spore-forming bacteria inhabiting the gut fluid of termites have been reported earlier (Breed et al. 1957, Breznak 1975). Arguments pointing against the spore nature of the described granules are, however, numerous: a. The small size; the granule receives only about 5-10% of the cell volume. b. The high electron density probably caused by the high P content. c. No developmental stages typical of endospore formation were detectable as, for example, formation of a forespore by a mother cell growing around a smaller daughter cell. At least, signs for a septum formation could be documented (Fig. 20) as it is typical, e.g., of the asexual exospore-formation of certain actinomycetes (Hardisson and Manzanal 1976). d. Setting free by pinching off instead of an autolytic process. Diverging types of spore-liberation, however, do exist, thinking for example of actinomycetes setting free their spores by rupture at the junction of the cell wall (Cross 1970) or budding exospores of the methan-consuming *Methylosinus trichosporium* (Schlegel 1981). e. Hatching 'spores' were not detected.

Other arguments support a spore nature: a. The uniform size b. A clear lining consisting of several layers including unit membranes. The layer between the inner and outer unit membrane could correspond to the spore cortex. c. The absence of smaller accumulating granules, which would be expected in case of reserve granules. Spore formation does not include stages with accumulation of smaller granules (Robinow 1960). The small dark particles with uniform diameter occasionally occurring in the adhering bacteria closely resemble virus particles (Ackermann and DuBow 1987, Southward and Southward 1991). d. The pin-like outgrowths appearing on the surface of the ingested granules could be compared to different kinds of appendages on the surface of clostridial spores. These arise as extrusions from the spore coat and may play a part in the germination process since these outermost structures are the first that come into contact with germinating agents (Walker 1970).

Supposing the granules were spores the intention could be the infection of the flagellate with a stage which is not susceptible to digestion as the normal bacteria. The spore-like granules could transform into endocytobiotic forms within the cytoplasm or nucleus. They need not be morphologically identical to the ectobiotic forms thus rendering their recognition more difficult. Bacteria with complex life cycles in connection with host cells do exist in protozoa, e.g., *Holospora* species but here the respective infective stages are probably different from spores (Görtz 1988, Tamura et al. 1971). There are several cytoplasmic and nuclear bacteria in *Joenia* whose origin is unknown leaving open a possible role of infection by spore-like forms.

The flagellate seems to be able to recognize granule-forming ectobiots since the ultrastructure of the attachment site changes. The diffuse layer of electron dense material underlying the contact zone transforms into a distinct, broken layer. However, it remains unknown whether the change in the bacterium evokes this alteration or vice versa.

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ACTA
PROTOZOOLOGICA

Contribution à la connaissance des ciliés psammophiles de la Baltique

Contribution to the Knowledge of Psammophilic Ciliates from the Baltic Sea

Anna CZAPIK et Janusz FYDA

Université Jagellone, Laboratoire d'Hydrobiologie, Kraków, Pologne

Résumé. Au cours du mois de juillet 1989 on a étudié les ciliés psammophiles littoraux dans le golfe saumâtre de Puck (la Baltique). On a déterminé 28 espèces. La densité de la population variait selon le lieu. A Władysławowo, où la pollution était la plus forte, elle dépassait 14000 individus dans un cm³ de sable.

Mots clés: ciliés, psammon.

Summary. Psammophilic ciliates in the littoral zone were studied in August 1989. Twenty eight species of ciliates were found. The highest concentration, exceeding 14000 ciliates per 1 cm³ of sand, was noted in the most polluted station at Władysławowo.

Key words: ciliates, psammon.

INTRODUCTION

Le travail que nous présentons ici est le supplément de nos études sur les ciliés psammophiles aux environs de Gdańsk (Czapik & Jordan 1976, 1977 a et b). En 1989 nous avons continué les recherches, en choisissant cette fois le golfe de Puck. Ce golfe fermé de l'ouest par la côte et du nord-est par le presqu'île Hel s'ouvre vers le sud dans le golfe de Gdańsk (Fig. 1). Dans cette zone la concentration du sel correspond à celle de la mer mais elle diminue légèrement vers le nord où le presqu'île forme avec la côte l'angle aigu. Dans cette région (Władysławowo, Chałupy) la salinité oscille entre 5-6‰.

Les rivages du presqu'île Hel sont bordés des plages couvertes de sable fin. L'eau semble transparente mais le sable examiné de près révèle une pollution assez forte. Si on enlève la couche superficielle du sable humide sur la plage de Jastarnia, on découvre d'abord une couche verte, remplie d'algues, plus bas une couche rose pleine de Rhodobactéries et finalement le sable gris-bleuâtre qui dégage l'odeur d'hydrogène sulfurique. La pollution augmente vers le nord, où le golfe est coincé entre la côte et le presqu'île. Ici, à Władysławowo on voit de grandes tâches roses sur le fond, tout près du rive. Cette pollution a deux sources: la canalisation locale et le courant venant de l'embouchure de la Vistule. Cette rivière apporte les eaux sales de plusieurs villes

Address for correspondence: Université Jagellone, Laboratoire d'Hydrobiologie, Oleandry 2a, 30-063 Kraków, Pologne.

Tableau 1

Liste des espèces trouvées dans le sable littoral

	Espèce	1	2	3	4	5	6
1	*Anigsteinia clarissima* (Anigstein)	+	–	+	+	–	–
2	*Aspidisca* sp.	–	+	+	+	–	–
3	*Cardiostomatella vermiforme* (Kahl, 1928)	–	+	–	–	–	–
4	*Chlamydodon mnemosyne* Ehrb.,1837	–	+	–	–	–	–
5	*Cinetochilum* sp.	+	+	–	+	–	–
6	*Coleps* sp.	–	–	+	+	+	–
7	*Condylostoma patulum* Clap. et Lach., 1958	–	+	–	+	–	–
8	*Condylostoma remanei* Spiegel, 1926	–	–	+	–	–	–
9	*Condylostoma rugosum* Kahl, 1930	–	+	+	–	–	–
10	*Dexiotricha* sp.	–	–	+	+	–	–
11	*Diophrys appendiculata* (Ehrb., 1838)	–	+	–	+	–	–
12	*Diophrys scutum* Dujardin, 1847	+	+	+	+	–	–
13	*Euplotes* spp	+	+	+	+	–	–
14	*Frontonia* spp	+	+	+	+	–	–
15	*Glaucoma* sp.	–	+	–	–	–	–
16	*Pseudokeronopsis decolor* (Wallengren, 1890)	–	+	–	–	–	–
17	*Lacrymaria olor* O.F. Muller, 1776	–	+	–	–	–	–
18	*Lacrymaria* sp.	–	–	+	+	–	–
19	*Litonotus anguilla* Kahl, 1930	+	+	+	+	+	–
20	*Loxophyllum levigatum* Sauerbrey, 1928	–	–	+	–	+	–
21	*Loxophyllum verrucosum* (Stokes, 1893)	–	+	+	+	–	–
22	*Mesodinium pulex* Clap. et Lach. 1858	–	+	+	–	–	–
23	*Metopus contortus* Quennerstedt, 1867	+	–	–	–	–	–
24	*Nassula labiata* Kahl, 1933	+	–	–	–	–	–
25	*Oxytricha* spp	+	+	–	+	–	–
26	*Paramecium balticum*	+	–	–	+	–	–
27	*Paraspathidium longinucleatum* Czap., Jor,1976	+	+	+	–	+	–
28	*Pleuronema coronatum* Kent, 1881	+	+	+	+	+	–
29	*Prorodon discolor* Echrb., Blochm, Schew.	+	–	+	–	–	–
30	*Prorodon raabei* Czapik, 1965	+	–	–	–	–	–
31	*Pseudouroleptus* sp.	+	+	+	+	–	–
32	*Sonderia sinuata* Kahl, 1930	+	–	+	–	–	–
33	*Spathidium* sp.	+	–	–	–	–	–
34	*Spirostomum teres* Clap. et Lach.,1859	+	+	+	+	–	–
35	*Stentor* sp.	–	–	–	–	+	–
36	*Strombidium* sp.	–	–	+	–	–	–
37	*Trachelonema oligostriata* Raikov, 1962	–	–	+	–	–	–
38	*Tracheloraphis margaritatus* (Kahl, 1930)	–	+	+	+	+	–
39	*Tracheloraphis phoenicopterus* (Cohn, 1866)	–	+	+	–	–	–
40	*Trachelostyla pediculiformis* (Cohn, 1866)	–	+	+	+	–	–
41	*Uronema marinum* Dujardin 1841	+	+	+	+	–	–
42	*Vorticella* spp	–	+	+	+	+	–
43	*Zoothamnium* sp.	–	–	–	+	–	–

LA BALTIQUE

Fig. 1. Le golfe de Puck

qui jusqu'ici n'ont pas de station d'épuration (entre autres Varsovie). Une partie de ces eaux sales déversées par la Vistule dans le golf de Gdańsk pénétre jusqu'au golf de Puck.

MATÉRIEL ET MÉTHODES

Les prélèvements du sable submergé pris sur les plages du golfe au cours du juillet 1989 ont été d'abord examinés "in vivo" au laboratoire; les espèces trouvées en nombre suffisante ont été fixées et imprégnées à l'argent selon méthode de Wilbert (1975).

Les prélèvements quantitatives ont été pris à l'aide d'une tube en plexiglass de 24 mm de diamètre. On mettait la couche du sable d'un cm d'épaisseur dans un bocal et on la diluait avec 50 cm^3 de l'eau filtrée, puisée du même endroit. On agitait fort le prélèvement pendant une minute et ensuite on prenait de l'eau avec une pipette automatique. Les ciliés dont la longueur dépassait 100 µm étaient comptés dans 10 gouttes de 50 µl, et les espèces plus petites dans 25 µl. Les résultats ont été ensuite calculés pour 1 cm^3 de sable.

RÉSULTATS ET DISCUSSION

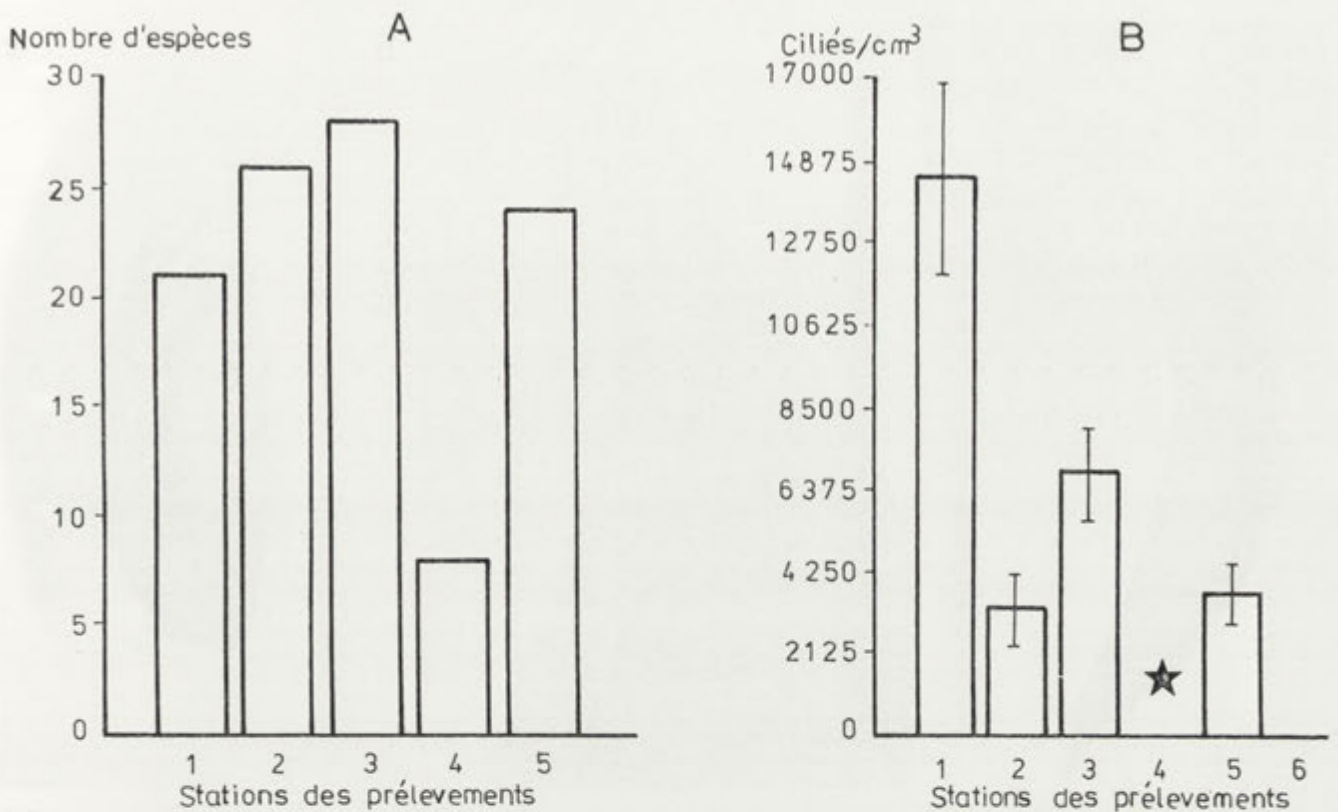Parmi les représentants de 43 genres que nous avons trouvés on a distingué 25 espèces qui formaient une

Fig. 2. A - Nombre des espèces trouvées à chaque station.   B - Nombre des ciliés dans un cm³ du sable. La ligne mince marque l'écart type (+,- ET), * manque de données. 1. Władysławowo, 2. Chałupy, 3. Kuźnica, 4. Jastarnia, 5. Jurata, 6. Hel

population dont la majorité se nourissait des algues ou des bactéries; les espèces carnivores appartenaient aux genres *Lacrymaria* et *Loxophyllum* (Fig. 5). Parmi les espèces microphages c'étaient *Pleuronema coronatum* (Fig. 8) et *Diophrys scutum* (Fig. 14) qui étaient les plus fréquentes: on les rencontrait partout. Par contre, *Metopus contortus* (Fig. 12), considéré comme index de la présence du hydrogène sulfurique, a été trouvé seulement à Władysławowo où il y a plein de Rhodobactéries dont ce cilié se nourrit.

Parmi les ciliés algivores les plus fréquentes étaient les espèces appartenant aux genres *Condylostoma* (Fig. 11) et *Tracheloraphis* (Figs. 3,4). En principe ces ciliés se nourissent de Diatomées et Phytoflagellés (Fenchel 1968) mais à Jurata on a observé une nombreuse population de *Tracheloraphis* qui avalaient les petits ciliés du genre *Cyclidium* dont fourmillaient les algues pourrissants sur le sable. Il faut encore noter que dans un prélèvement de Władysławowo nous avons trouvé *Nassula labiata* (Fig. 6). C'est Kahl (1930) qui a décrit cette petite espèce de couleur brune, mais sans

donner les détails morphologiques. Il l'a découverte dans le milieu saprobe de la mer Baltique. En 1972 Borror a redécrit ce cilié qu'il a trouvé dans les marais salés de New Hampshire. Il paraît que cette espèce, bien que rare, est cosmopolite.

Le nombre des espèces variait selon de lieu (Fig. 2A). La faune la plus riche se trouvait à Kuźnica, où la pollution était moyenne. Par contre, le lieu le plus pauvre, c'était la plage du village Hel où nous n'avons rencontré aucun protozoaire. Les seuls animaux qui y vivaient étaient représentants d'une petite espèce des Turbellariés qui nageaient parmi les grains du sable avec une rapidité surprenante. Cette pauvreté qui semblait bizarre s'est expliqué quand nous avons appris qu'il y a deux ans on avait artificiellement élargi la plage d'Hel, en y ajoutant une grande quantité du sable apporté de la côte. Au cours de cette période les animaux n'ont pas encore arrivé à peupler ce terrain nouveau.

La densité de la population des ciliés variait suivant le lieu (Fig. 2B); elle était la plus grande à Władysła-
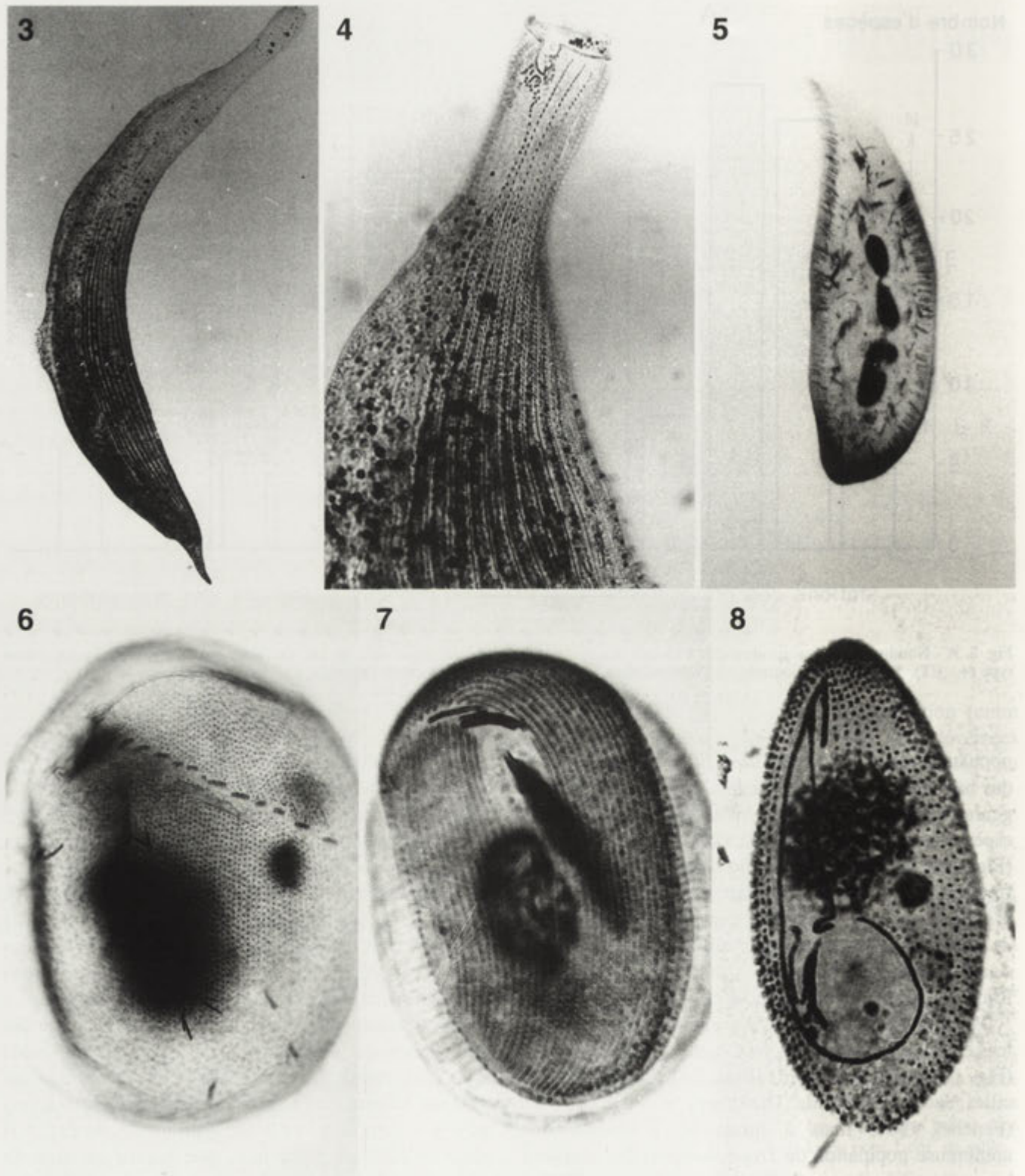
Figs.3,4. *Tracheloraphis margaritatus.* 3 - vue générale du cilié (x 160),4 - la bouche (x 708). 5. *Loxophyllum verrucosum* (x 278). 6. *Nassula labiata* (x 697). 7. *Chlamydodon mnemosyne* (x 1357). 8. *Pleuronema coronatum* (x 1101)

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Figs.9. *Sonderia sinuata* (x 1302). 10. *Cardiostomatella vermiforme* (x 785). 11. *Condylostoma patulum* (x 371). 12. *Metopus contortus* (x 1280). 13. *Strombidium* sp. (x 1166). 14. *Diophrys scutum* (x 893)

wowo où le nombre des ciliés dans un cm^3 de sable pris de la couche superficielle dépassait 14000 individus. Dye (1979) a trouvé 695 - 1340 ciliés dans un cm^3 du sable de la plage à Port Elizabeth. Selon Fenchel (1969) la densité de ciliés est très variée; elle peut dépasser 4000 individus/cm^2. Les chiffres que nous avons obtenu sont pareils sauf ces à Władysławowo, où en raison de la pollution très forte le nombre des ciliés est extrêmement élevé.

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ACTA PROTOZOOLOGICA

Culture and Preservation of Naked Amoebae

Lidia V. KALININA* and Frederic C. PAGE**

*Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia; **18 The Coppice, Impington, Cambridge, United Kingdom

Summary. Methods of culturing and preserving strains of free-living naked amoebae (freshwater, soil, marine) belonging to the classes Lobosea, Heterolobosea, Caryoblastea and Falosea are presented. These include general principles of isolation, axenicisation, maintenance and cryopreservation as well as detailed methods for many genera and species. Data are drawn from the authors' own work and from the literature.

Key words. Amoebae, Caryoblastea cryopreservation, culture, Filosea, Heterolobosea, Lobosea.

CONTENTS

Address for corespondence: L.V.Kalinina, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky Avenue. 4, 194064 St. Petersburg, Russia.

1. CULTURE AND MAINTENANCE

1.1. INTRODUCTION

This chapter presents general principles and detailed methods for culturing free-living organisms classified in the following groups of Rhizopoda: class Lobosea, subclass Gymnamoebia; class Heterolobosea, order Schizopyrenida; class Caryoblastea; class Filosea, family Nucleariidae (Page 1987). The organisms include species from freshwater, soil and salt water. Further information on the organisms themselves can be found in the taxonomic publications of Page (1983, 1988, 1990). For methods of culturing obligately parasitic amoebae the reader should consult the parasitological literature.

In order to cultivate organisms intelligently, their life cycles must be understood. Besides the trophozoite or vegetative stage, many species produce cysts or resting stages, which may remain viable only a few days but are often viable for years, providing a convenient method of storing the strain. Some organisms, mostly members of the Heterolobosea, have a temporary flagel-

late stage. Sexual processes are known for very few amoebae.

Workers culturing amoebae should be aware that certain normally free-living specie; have been found to be potential pathogens of humans and laboratory animals. *Naegleria fowleri*, occurring in naturally and artificially warmed waters (e. g., swimming pools, effluent from cooling systems of power stations), may invade the brain through the nose and cause the fatal disease primary amoebic meningo-encephalitis. One other species of the same genus, *N. australiensis*, is pathogenic for laboratory animals, but no human cases due to this species have been reported. Strains of *Acanthamoeba*, especially *A. culbertsoni*, have also been reported as pathogens of the central nervous system. Several species or *Acanthamoeba* have been implicated in keratitis of the cornea, in some cases with loss of the eye.

Strains of potentially pathogenic free-living amoebae (amphizoic amoebae) should therefore be handled with appropriate precautions. Infections with *N. fowleri* are caused by the introduction or contaminated water into the nose, usually while swimming. No infections or laboratory workers with either *Naegleria* or *Acanthamoeba* are known. *N. fowleri* is unlikely to grow out in collected material incubated at 18-22°C. The species or species-group *N. gruberi* is common in nature and appears to be non-pathogenic. *Acanthamoeba* is the most commonly isolated genus of naked amoebae. Avoidance of contact with *Acanthamoeba* is impossible in everyday life, and strains from corneal infections do grow at 18-22°C.

1.2. GENERAL PRINCIPLES

Culture methods differ according to the needs or the organisms and the purposes for which they are cultured.

Media may be liquid, agar-based or biphasic that is, agar with a liquid overlay. The largest amoebae are cultured in liquid, though a few fairly large ones (Thecamoebidae) are usually grown on agar. Liquid media are also used for axenic culture and for some medium-sized amoebae, e. g., *Mayorella*. Many small amoebae become more numerous on an agar surface than they do in liquid, even though they naturally occur in water.

The inorganic liquid used to make either liquid media or agar may vary widely in tonicity. For most freshwater amoebae it is a very dilute solution of salts, usually buffered, in distilled water. However, unpolluted natural water with little organic content can also be used for many purposes. A few species are grown in media which are approximately isotonic with mammalian body fluids. For marine amoebae seawater is used. The seawater should be unpolluted and is therefore perhaps best collected at sea far from land. Its salt content may be about 30-35‰, though we have found that many amoebae collected near the shore grow best at a salinity of about 25-27‰ (here called 75% seawater), and amoebae from waters of low salinity require more dilute media. Special attention is necessary for the salinity requirements of amoebae from various parts of the Baltic. "Artificial seawater" may be prepared from inorganic salts but may lack some inorganic or organic components. On the other hand, commercially produced "artificial seawater" sometimes appears richer in nutrients than is natural seawater and may result in faster growth and therefore necessitate more frequent subculturing.

The content or dissolved organic nutrients in a medium varies from complete absence in the very dilute salt solutions used for some large amoebae to the protein-rich liquid media described in the next section. An intermediate class includes infusions made from grains or leaves. Sometimes the particulate matter is left in the medium, but often it is filtered out after boiling. Diverse grains were found suitable for various protozoa (Page 1981), but the most useful are lawn-grass seed, barley and wheat. Uncooked grains of polished rice are added to some media. The nutrients in infusions provide food for the bacteria upon which the amoebae feed, though it is possible that they also contribute directly to the diet of the amoebae. It is important not to make the media for cultures containing bacteria too rich.

In axenic cultures the dissolved nutrients serve as the only food for the amoebae. Such nutrients include proteose peptone, serum, glucose, malt extract and yeast extract.

The particulate food of cultured amoebae usually consists of living organisms. Bacteria are the most common food, especially of smaller amoebae. Cultures of amoebae feeding on bacteria are therefore grown in or on media with a relatively low content of dissolved nutrients so that the bacteria do not overgrow the amoebae. Often amoebae are feeding on unidentified bacteria which have accompanied them from their natural habitat. Small freshwater and soil amoebae are most commonly grown on non-nutrient agar streaked

with *Escherichia coli* which have been grown on ordinary bacteriological nutrient agar.

Other organisms which serve as food include other protozoa, such as *Tetrahymena* or even smaller amoebae. If these organisms have been grown in a rich medium (e. g., *Tetrahymena* in proteose peptone yeast medium), they must be washed by centrifugation before being added to the amoeba cultures. Algae, including fragmented filaments of blue-greens (cyanobacteria), are used as food for a few species.

All media should be sterilised during or after preparation, and all culture vessels should be sterilised, even if the cultures will contain unidentified accompanying bacteria. In this way contamination with organisms such as micrometazoa (nematodes, rotifers) and algae is minimised. Agar cultures and axenic liquid cultures should be handled with sterile precautions, though completely sterile handling is usually unnecessary with large amoebae in liquid (e. g., *Amoeba, Chaos*) and cultures in infusions or seawater may be examined with the cover removed if an inverted microscope is not available. Shelves and cupboards should be cleaned frequently to minimise the possibility of contamination with mites.

Attention to physical conditions is necessary for successful culture.

pH: The optimum for most freshwater and soil amoebae appears to be a short distance to either side of 7.0. The pH of marine media is usually higher but has not been systematically investigated.

Temperature: Most free-living amoebae grow well at 19-20°C. Some species grow well at temperatures to 30° and higher. Potential pathogens are usually grown at 37°, though some will do well at 44° or 45°C. However, temperatures of 25° or above can be lethal to cultures of some species, especially larger amoebae and marine amoebae.

Light: Cultures should never be subject to direct sunlight, and liquid cultures other than axenic cultures are best kept in the dark to prevent growth or algal contaminants. However, *Mayorella viridis* and cultures in which it is thought that algae may be serving as food organisms require a normal light/dark cycle.

Oxygen: Most free-living amoebae are aerobic. The exceptions include *Pelomyxa palustris, Entamoeba moshkovskii* and *Phreatamoeba balamuthi*. Often the need of these organisms for a low oxygen tension can be satisfied by growing them in tubes, in which they are found at the bottom rather than near the surface, where axenic *Acanthamoeba* for example, is found. When

amoebae are grown in liquid in culture dishes, the liquid should be no more than 10-12 mm deep in order to permit adequate oxygenation.

Evaporation: This must be minimised in order to prevent concentration of solutes or even drying out of the medium. At the same time oxygen exchange should not be prevented. These conditions can be met by closing each culture vessel, whether a tube or a dish, with a strip of polythene film (clingfilm) covering the junction between cover and vessel. This method also reduces the chance of contamination by mites and airborne fungal spores. Petri dishes should be unvented or singly vented, never triply vented.

1.3. METHODS

1.3.1. Composition of media

The quality of water used for media has already been discussed. If certain other components are not available, possible substitutes should be tested.

1.3.1.1. Dilute salt solutions

Modified Neff's amoeba saline (AS)

Prepare stock solutions of the following quantities of salts, each in 100 ml of glass-distilled water: 1.2 g NaCl, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot H_2O$, 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4. Final solution: 10 ml of each stock solution + 950 ml of glass-distilled water.

Chapman-Andresen's modified Pringsheim's solution (MP)

Prepare stock solutions of each of the following salts, each in 100 ml of glass-distilled water: 20.0 g $Ca(NO_3)_2 \cdot 4H_2O$, 2.0 g $MgSO_4 \cdot 7H_2O$, 2.0 g $Na_2HPO_4 \cdot 2H_2O$, 2.6 g KCL, 0.2 g $FeSO_4 \cdot 7H_2O$. Final solution: 1 ml of each stock solution + 995 ml of glass-distilled water.

Modified Chalkley's solution (MCh)

Prepare stock solutions of each of the following salts, each in 100 ml of glass-distilled water: 8.0 g NaCl, 0.04 g $NaHCO_3$, 0.04 g KCl, 0.16 g $Ca(H_2PO_4)_2 \cdot H_2O$. Final solution: 1 ml of each stock solution + 996 ml of glass-distilled water.

Prescott's & Carrier's solution (PC)

Prepare 2 stock solutions, each in 1 l of glass-distilled water: (A) 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 1.0 g $CaCl_2$ and 1.0 g NaCl. (B) 0.36 g $CaHPO_4$. Final solution: 10 ml of each stock solution + 980 ml of glass-distilled water.

Prescott's & James's solution (PJ)

Prepare 3 stock solutions, each in 100 ml of glass-distilled water: (A) 0.433 g $CaCl_2 \cdot 2H_2O$ and 0.162 g KCl. (B) 0.512 g KH_2PO_4. (C) 0.280 g $MgSO_4 \cdot 7H_2O$. Final solution: 1 ml of each stock solution + 997 ml of glass-distilled water.

1.3.1.2. Modified Føyn's Erdschreiber (Erds)

Prepare the following solutions soil extract: Mix 1 kg garden soil (not recently fertilised) and 1 l glass-distilled water. Adjust pH to 8.0 with NaOH. Autoclave for 1 h at 15 lbs pressure. The supernatant liquid is the soil extract. Stock solutions: 20 g $NaNO_3$ in 100 ml of glass-distilled water. 1.2 g Na_2HPO_4 in 100 ml of glass-distilled water. Final solution: 900 ml of filtered seawater + 100 ml of soil extract + 1 ml of each stock solution.

1.3.1.3. Infusions

Cerophyl infusions

Cerophyl is a powder prepared from dried cereal leaves, manufactured in the USA and used widely for infusions in which ciliates are cultured. Similar preparations may be available in other countries, and workers may wish to devise their own preparations. Problems include nutritional adequacy and freedom from agricultural chemicals.

Cerophyl/Prescott's & James's infusion (CP) for freshwater and soil amoebae: Boil 0.5-1 g of Cerophyl in 1 l of PJ solution for 5 minutes, filter out the particles, and restore volume to 1l with distilled water.

Cerophyl/75% seawater solution (C75S) for marine amoebae: Use 75% seawater (seawater diluted to a salinity of 25-27‰) instead of PJ.

Other infusions

Lawn-grass seed has often been used. It should be free of chemicals such as fungicides. Boil 2 g of seeds in AS,PJ or another suitable liquid for 5 minutes. Pour the infusion into culture dishes, with a few grains in each dish, and discard the excess seeds. Grass-seed infusion in seawater has not been tested.

Barley, wheat and maize kernels are less useful for amoebae than for ciliates. Infusions of barley or wheat are prepared in the same way as grass-seed infusions. For maize infusion, dried kernels are boiled 10 minutes, after which a hole is torn in the side of the kernel. No more than 1 or 2 maize kernels should be put into a culture dish. Cultures in maize infusion may soon contain excessive bacteria. The quantity of grains used in preparing infusions may be varied.

It should be emphasised that the polished rice grains used in cultures of larger amoebae are not boiled. They may be surface-sterilised by *rapid* passage through a flame, then put into the appropriate liquid, usually 2 or 3 grains in a culture dish with a diameter of 50 mm, more in a larger dish. The media thus prepared are not actually infusions. However, rice grains (2 per 50 mm culture dish) are usually added to CP or C75S infusion, giving better cultures than the infusion alone or salt solutions alone, especially with *Mayorella*, which is often cultured in CP or C75S with rice.

1.3.1.4. Agar

After preparation agar media should be autoclaved and then poured into sterile 9-10 cm petri dishes to a depth of 3 or 4 mm. Such agar plates may be stored a week before use under clean conditions which minimise the possibility of contamination by mites or fungi.

Non-nutrient agar (NNA)

15 g of a non-nutrient agar powder such as Oxoid No. 1 or Difco Bacto-Agar is added to 1 l of modified Neff's amoeba saline (AS), which is then brought just to a boil.

Cerophyl/Prescott's & James's agar (CPA)

Make as NNA but substitute CP infusion for the AS. Possibly other saline solutions such as AS would be just as useful as PJ in making the Cerophyl infusion for this agar.

Grass-seed agar (GSA)

If Cerophyl is not available, this medium can be used instead of CPA. Make as NNA but substitute grass-seed infusion for the AS. When pouring try to get approximately the same number of seeds in each petri dish, and try to distribute them fairly evenly in each dish. All the grass seeds used to make the infusion should be left in the agar.

Cerophyl/75% seawater agar (C75SA)

Make as CPA but use filtered seawater diluted with glass-distilled water to a salinity of approximately 25-27‰ . If Cerophyl is not available, use the next medium. Seawater agar must be mixed thoroughly as it is poured into the petri dishes.

Grass-seed/75% seawater agar (GS75SA)

Make a grass-seed infusion in 75% seawater, and add 15 g of a non-nutrient agar powder.

Malt extract/yeast extract/75% seawater agar (MY75SA)

This is the principal medium for small marine amoebae. In 1 l of filtered 75% seawater dissolve 0.1 g malt extract, 0.1 g yeast extract and 15 g of a non-nutrient agar powder.

1.3.1.5. Protein-rich liquid media

These are used mostly for axenic cultures, though some cultures grown in Jones's medium contain bacteria.

Proteose peptone glucose (PPG)

Dissolve 10 g proteose peptone and 18 g glucose in 1 l AS liquid. Autoclave in culture tubes.

Chang's serum/casein/glucose/yeast extract medium (SCGYEM)

Isoelectric casein, 10.0 g; glucose, 2.5 g; yeast extract, 5.0 g; Na_2HPO_4, 1.325 g; KH_2PO_4, 0.8 g; foetal calf serum, 100 ml; glass-distilled water, 900 ml. When the inoculum may contain bacteria, add 200 µg/ml penicillin and the same quantity of streptomycin. Autoclave in culture tubes.

Jones's medium

Prepare buffered saline: $Na_2HPO_4 \cdot 12H_2O$, 2.65 g; KH_2PO_4, 0.41 g; NaCl, 7.36 g; glass-distilled water, 1 l. Final pH 7.2. The final medium consists of 850 ml buffered saline, 50 ml horse serum and 100 ml of 1% yeast extract solution. Sterilise the final medium by filtering, pour into sterile tubes, and add a pinch of sterile rice starch to each tube. Rice starch can be sterilised by heating it at 150 °C for 2 hours.

1.3.2. Culture and isolation

Samples should be collected from natural habitats with sterile equipment in order to prevent contamination with cyst-forming amoebae and other organisms from the collecting equipment, though this precaution is not necessary if one is trying to isolate only large amoebae such as *Amoeba* and *Chaos*. Collected material should be handled with sterile precautions until samples have been inoculated into appropriate media.

Samples of collected material should be examined microscopically soon after collection to detect any amoebae which may not grow out in culture or to find large amoebae for immediate cloning. Samples should also be inoculated into or onto the appropriate medium, according to the amoebae which are sought. The appropriate medium for each genus or species is given under the heading 'Systematic survey of culture methods' below. In general the collected material should be enriched for amoebae, but for *Amoeba* or *Chaos* it is best to clone directly from the collected water with a fine pipette. The best initial media for enriching the populations of other amoebae are: (1) For amoebae which when purified will be grown on either NNA with *Escherichia coli* (NNA/Ec), on CPA or on GSA, inoculate about 1 ml of liquid (collected water, or soil + liquid) onto either CPA or GSA, not immediately onto NNA/Ec. (2) For marine amoebae which will be grown on MY75SA, inoculate collected water and, if present, bits of algae onto either C75SA or G75SA, not immediately onto MY75SA, which may produce too much bacterial growth. (3) For freshwater, soil or marine amoebae, e. g., *Mayorela* which will be grown in an infusion, in seawater or in Erdschreiber, inoculate several millilitres of collected water into a culture dish containing the medium, after adding 2 or 3 grains or sterile polished rice if the culture instructions so specify. It may be useful also to inoculate some collected material into a small culture dish containing a thin layer of CPA or GSA overlaid with liquid to a depth of 2 or 3 mm in order to enrich for any medium-sized amoebae which can grow under those conditions.

The collected material is likely to contain food organisms for the amoebae which are to be isolated. But when the initial cultures have grown out, each may well contain more than one species of amoebae. Clones must be established in order to ensure the presence of only one species of amoebae. Clonal strains are also desirable because all members of a clone are, at least when the strain is young, genetically identical.

Mixed cultures on agar can be used as sources or clones approximately a week after inoculation of the collected material, though an incubation period of 10-14 days may be better for amoebae which multiply slowly, such as *Thecamoeba*. Mixed cultures in liquid should be incubated about 14 days before isolation of clones.

1.3.2.1. Cloning

The method of cloning depends on the size of the amoebae and the presence or absence of cysts.

***Amoeba*, *Chaos* and other large amoebae grown an liquid:** Using a sterile micropipette, a single amoeba is removed from either a mass culture or a small quantity or liquid and put into a chamber (microaquarium) in a subdivided plastic plate or Terrasaki dish, each chamber containing a small quantity of the appropriate liquid, e. g., PC. A drop of washed *Tetrahymena* is added to the chamber. When all chambers are filled, put the plate into a moist chamber. Change the culture medium daily, or transfer the amoeba(e) to a new chamber. When more than 32 amoebae are present in any chamber, transfer that clone to a culture dish.

Cysts: For most species which form cysts, it is best to start each clone with, a cyst, because cysts are more easily seen and less likely to be damaged than active amoebae. Wash the agar surface of the mixed culture with 0.5-1 ml of AS or PJ, and streak some of this suspension onto NNA with a bacteriological loop. Find an isolated cyst, and with a sterile fine scalpel cut out a block of agar bearing that cyst but no other cysts or trophic amoebae and put it on a fresh surface of the appropriate agar. It is easier to cut out a small block of agar (1 or 2 mm square) if one first finds the cyst, then marks it with a bright spot of light from the condenser, then lowers the stage to give more room for working. The scalpel may be sterilised by dipping it in alcohol, burning off the alcohol and permitting the scalpel to cool. Some bacteria from the natural habitat will accompany the cyst on the block of agar. If the medium onto which the block is inoculated is CPA or GSA, these bacteria will multiply and provide food for the amoebae.

Small or medium-sized trophic amoebae: If a species does not form cysts readily or at all, obviously clones must be started with trophic amoebae. Such species include all marine amoebae and freshwater/soil species or *Mayorella*, *Vannella* and *Thecamoeba*. Information on occurrence of cysts is given under 'Systematic survey of culture methods', but the occasional cysts of Amoebidae are not suitable for this method of cloning. This method is recommended also for larger and medium-sized cyst-forming amoebae such as *Sappinia* and *Deuteramoeba*. Even amoebae which are normally cultured in liquid medium can be cloned by cutting from an agar surface if they will survive some time on agar.

Make a suspension from the agar surface by adding 0.5-1.0 ml of the appropriate liquid (e. g., CP or 75% SW) to the mixed culture, and place 3 drops about 1 cm apart on one side of a fresh plate or agar (Fig. 1a). Tilt the plate so that the drops run parallel to each other across the agar. Then let the plate stand a few hours or over night until some amoebae have migrated from the parallel streaks and are well separated from other amoebae (Fig. 1b). Cut out blocks each bearing only one amoeba to start clones, as described for cloning cysts. If the amoebae to be cloned are large or medium-sized and relatively few, it may even be possible to pick out single amoebae immediately after inoculating the cloning surface, without waiting for them to migrate. In that case cut out a block bearing only one large amoeba but try to include also any small amoebae or other small protists that are present and may serve as food for the large amoebae. If the accompanying organisms are found not to serve as food, they can be eliminated later. If the amoebae to be cloned are in mixed culture in liquid, simply inoculate 3 drops of this liquid onto the agar cloning surface as described for amoebae from agar.

It is advisable to isolate at least 10, preferably more, potential clones at one time from each mixed culture, because not all will grow out and the clones may prove to belong to two or more species. Examine the attempted clones after about 5 days and continue to examine them for some time thereafter before discarding, up to 2 or 3 weeks for medium-sized and larger amoebae.

The clones isolated by the methods described will contain unidentified bacteria, which may serve as food for the amoebae, and clones of medium-sized amoebae may also contain protists. Further purification is often possible, but one must take care not to eliminate essential food organisms.
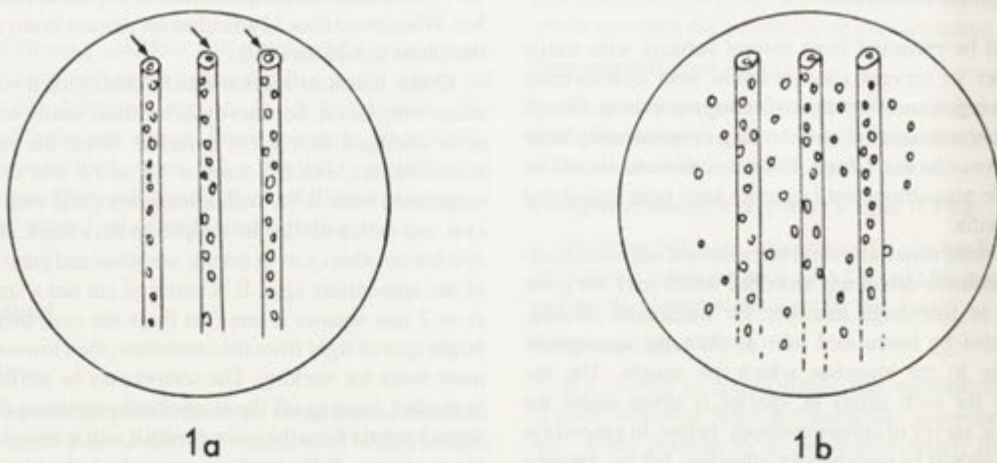
Fig. 1. Cloning by migration of amoebae. a) Isolation plate immediately after inoculation. Arrows indicate positions of 3 drops from which streaks have been produced. All amoebae are still within streaks. b) Isolation plate the next day. Some of the amoebae which have migrated far enough from the streaks can be used as clone-founders

1.3.2.2. Monoxenicisation and Axenicisation

Monoxenicisation is the production of cultures containing only one other species besides the one being cultured. Most commonly freshwater and soil amoebae are brought into monoxenic culture with *Escherichia coli*, on which they feed. The preferred method of monoxenicising freshwater and soil amoebae growing on NNA is ilustrated in Fig. 2a. Make a single streak of *E. coli* across the diameter of an NNA plate. Inoculate a block of agar bearing amoebae from a clonal culture at one end of the streak (q). Over a period of approximately a week (depending on species) the amoebae will multiply and migrate to the other end or the streak (w). As soon as the *first* amoebae have reached the w end, inoculate a block of agar bearing amoebae from that end at one end of a streak on a fresh plate. After 3 or 4 such serial cultures, other accompanying bacteria should have been eliminated, but the purity of the culture should be tested bacteriologically.

Axenicisation is the production of cultures containing no other organisms but the species being cultured. Amongst free-living amoebae, only *Acanthamoeba* spp. and *Naegleria* spp. are commonly grown axenically. It is more difficult to axenicise some species of these genera than others, and some strains of *Acanthamoeba* have never been axenicised.

Amoebae can be axenicised by the use of antibiotics (penicillin and streptomycin), but inhibition of bacteria does not always lead to their complete isolation. Migration methods of axenicisstion depend on the ability of amoebae to migrate to a bacteria-free area of an agar surface, from which they are then taken before later amoebae have brought bacteria to that area. Sometimes axenicisation as achieved by inoculating amoebae onto agar with killed bacteria. A method which uses live bacteria and has proved successful for establishment of *Acanthamoeba* clones is shown in Fig. 2b. Make a crescent-shaped streak of *E. coli* on one side of an NNA plate. Place a drop of amoeba suspension between the streak and the side of the plate. The
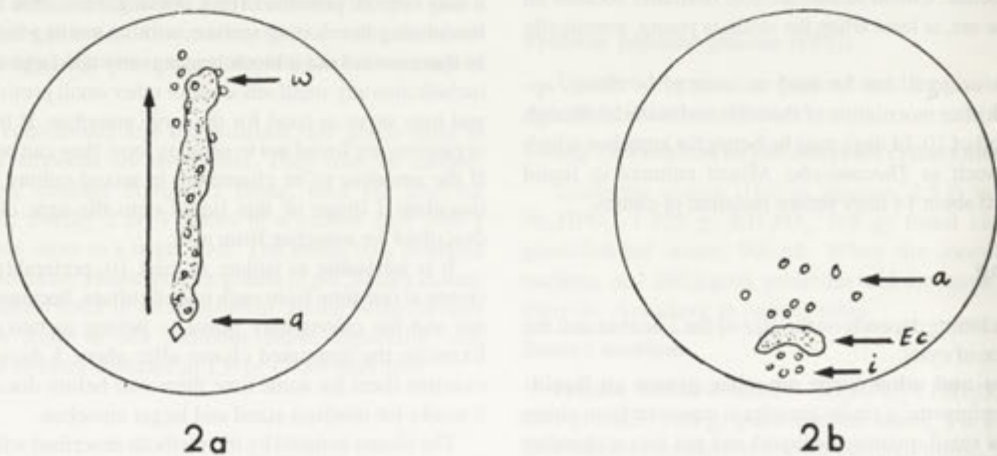

Fig. 2. Purification by migration. a) Amoebae inoculated on block of agar at q have entered bacterial streak, multiplied and migrated to ω, from which they can be transferred to a fresh plate. Several serial migrations through streak of *E. coli* should produce monoxenic cultures. b) Amoebae inoculated at *i* enter crescent-shaped streak of *E. coli*, multiply and migrate further. The first ones at *a* may be free of all bacteria and can be used as inoculum to start axenic cultures

amoebae will move into the bacteria, feed and multiply, then migrate further. The *first* amoebae to migrate 1 cm or more beyond the streak may be free of bacteria. It is essential to transfer such amoebae on an agar block as soon as possible into liquid medium for axenic culture, several amoebae into each tube. After a few days, discard any cultures which are grossly contaminated with bacteria. After 2 or 3 weeks, take a drop of liquid near the top of the medium and examine microscopically for amoebae. If amoebae are present but no bacteria are seen, let the culture grow a week or two longer, then test bacteriologically for purity. It is advisable to inoculate 10-20 tubes each time one attempts axenicisation, because some will be contaminated and some will not grow out.

1.3.3. Maintenance of cultures

1.3.3.1 Subculturing

Strains of amoebae must be subcultured, i. e., transferred to fresh medium, at regular intervals, which vary according to species. It is advisable to make at least 3 new cultures each time, in case some become contaminated or do not grow out, though with most strains one can go back to older cultures if the new ones fail. Approximate intervals between subcultures for maintenance are given in the instructions for each genus or species in the following section. Subculturing should be carried out while viable cells are fairly numerous. It may be possible to extend the intervals between transfers somewhat, but cautious tests should be carried out first.

Amoebae in liquid culture are transferred with pipettes. If they are in protein rich medium (usually axenic), the culture should be agitated with the pipette and a pipette-full transferred to the new medium, observing sterile precautions. For non-axenic cultures in liquid in 50 mm culture dishes the following procedure has proved very useful: Into a sterile culture dish pour fresh medium equal in quantity to the parent culture. Then with a pipette transfer the entire parent culture into a new dish, thus mixing it with the fresh medium,

and then transfer half of the mixed liquid back into the old dish. This procedure provides a large inoculum for the daughter culture and rejuvenates the parent culture.

Amoebae in agar cultures are usually subcultured simply by transferring a block of agar from the parent culture to a fresh agar surface. If the medium is NNA, make a straight streak or *E. coli* on the fresh surface before adding the amoebae, and place the block at one end. This prolongs the life of the culture, since the amoebae must reach the other end of the streak before exhausting their food supply. If transferring to CPA, GSA or MY75SA without an *E. coli* streak, place the block toward one side of the fresh agar surface. Before subculturing it is best to examine the parent culture by turning the culture dish over on the microscope stage, finding a suitable area of the culture, marking that spot on the bottom of the dish, removing the dish from the stage and cutting out the block or agar. The block is placed on the fresh agar with the side bearing the amoebae against the fresh surface.

If amoebae on agar multiply slowly or few are present, it is best to transfer them as a suspension by washing the agar surface with 1 ml of liquid, pipetting that suspension onto a fresh surface and tilting the plate to distribute the liquid. The purpose is to prevent bacteria overgrowing the amoebae (on CPA, GSA or MY75SA) and permit development of several foci of growth.

After agar plates have been inoculated, they can be incubated in either a normal or an inverted position. However, it is best to keep them in the normal position for at least the first few days, so that the agar block bearing the inoculum does not drop off or the suspension does not run down onto the cover of the plate.

1.3.3.2. Storage and preservation

Successful cryopreservation has been achieved for many strains of free-living amoebae. The methods are presented in section 2. Cryopreservation, with details in Table 1. The present section describes methods of storing organisms without freezing.

Table 1

Method	Cryoprotectant	Equilibration time and temperature	Ampoule type	Freezing programme
1	5% DMSO	30 min, RT	Plastic	From 25 °C cool cells 10 °C/min to - 2 °C, then 1 °C/min to - 45 °C, then plunge them rapidly into LN
2	5% DMSO	30 min, RT	Glass, plastic	Place ampoules at bottom of - 55 °C freezer for 1 hour, then plunge them rapidly into LN
3	5% DMSO	30-60 min, RT (optionally ice bath)	Glass, plastic	Standard freeze to - 40 °C, or 1 °C/min from 4 °C to - 40 °C, then plunge or override to - 150 °C
4	–	-	Shell vial	After drying at 25 °C for 14 days, followed by 1 week in vacuum desiccator at RT, place ampoules into vapour phase of LN and store at - 60 °C, - 70 °C
5	12% DMSO +6% sucrose	30 min, 4 °C, RT, 28 °C	Glass, plastic	Standard freeze to - 40 °C *, then pluge into LN
6	–	–	-	Single cells on aluminium substrate, plunge rapidly into slurry (mixture of solid and liquid N₂)

Abbereviations: DMSO, dimethyl sulphoxide; LN, liquid N_2; RT, room temperature.
* Test-tube cultures must be iced before centrifugation

Storage of strains during the interval between subculturings depends on the live history and growth characteristics of the strains.

Strains which form many cysts can often be stored for a long period on slopes of NNA in tubes closed with screw caps. The slopes should first be streaked with *E. coli* and inoculated with amoebae, then incubated at their normal growth temperature (see 1.4. Systematic survey of culture methods) long enough to permit multiplication and encystment. They can then be stored at a temperature of 6-9°C until time for the next subculturing. The great majority of such cultures are still viable after a year, but some require subculturing every 6 or even 3 months. Only a few free-living amoebae have cysts with a shorter period of viability, e. g., 6 or 8 weeks, and those strains should be treated in the same way as non-encysting amoebae. Cultures or some cyst-forming amoebae have proved viable after 5 years or more, but it is risky to leave them more than a year without subculturing. Investigations of the possibilities of cryopreservation of cyst-forming and other amoebae have involved only a few species.

For non-encysting strains such long storage is not possible, and we have not found it practical to store agar cultures of marine amoebae or most non-encysting freshwater and soil amoebae on slopes at low temperatures. Such strains are kept on agar plates between transfers. We have usually stored them at 18-22°C but storage at 15° or slightly lower should be attempted, once the cultures have been incubated long enough to become abundant. It should be kept in mind that populations of non-encysting amoebae remain active and need some food even at temperatures somewhat lower than the usual incubation temperature. Storage for more than 8 weeks is not recommended, and some species require more frequent transfers, though it is often possible in an emergency to inoculate from cultures older than 8 weeks.

Amoebae in high-protein liquid media, which are usually axenic, can also be stored at temperatures of 13-15°C, possibly even somewhat lower, after being incubated at their usual growth temperature. Our normal practice with axenic cultures of *Acanthamoeba* has been to subculture strains stored at those temperatures every two months, but further investigation is recommended with various intervals and temperatures for *Acanthamoeba*, *Naegleria* and *Phreatamoeba* in rich liquid media.

Large and medium-sized amoebae in low-nutrient liquid media such as saline solutions and infusions can be stored at 18-22°C, though a temperature of 18 or 19°C is recommended. In general these should be subcultured at intervals of a month or less; the best intervals are given in the "Systematic survey of culture methods".

Even when routines of storage and subculturing have been established, careful observation of changes in growth patterns or possible needs for changes in procedures is essential to prevent loss of strains. For example, encysting strains may surfer reduction or even loss of ability to encyst, so that they must then be treated as are non-encysting species. Strains may show a decline after years in culture not multiplying as rapidly or producing such large populations as they did soon after isolation. Such a decline may necessitate more frequent attention. In the case of marine amoebae on agar, such declines may be the result of a change in the accompanying bacterial flora on which the amoebae feed. Streaking or MY75SA with *E. coli*, which does not multiply much on that agar at 18-22°C, has proved successful in restoring the population level of some marine strains.

Exposure to high temperatures is lethal to many amoeba cultures. For some, such as marine amoebae on agar, prolonged exposure to temperatures above 25°C is fatal.

1.3.4. Systematic survey of culture methods

The amount of information given for each genus or species reflects knowledge of culture conditions, which is greater for some amoebae than for others. Workers may have to adapt the procedures to those which are possible for them. For example, if Cerophyl or a suitable substitute is not available, grass-seed infusion or grass-seed agar should be tested as possible substitutes.

Abbreviations used in this section:

AS	modified amoeba saline
C75S	Cerophyl infusion in 75% seawater
C100SA	Agar made with Cerophyl infusion in undiluted seawater
CP	Cerophyl infusion in PJ
CPA	CP agar
CP/PJ	CP diluted 1:1 with PJ
Cry	For cryopreservation see Table 1.
/Ec	*Escherichia coli* streaked on agar
Erds	modified Føyn's Erdschreiber medium
MP	Chapman-Andresen's modified Pringsheim's solution
MY75SA	malt extract/yeast extract/75% seawater agar
MY100SA	malt extract/yeast extract agar made with undiluted seawater
NNA	non-nutrient agar
PC	Prescott's & Carrier's solution
PJ	Prescott's & James's solution
PPG	Proteose peptone glucose medium
/r	polished, uncooked rice grains added to liquid
SCGYEM	Chang's serum/casein/glucose/yeast medium
st	temperature (°C) at which cultures are stored after growing out
tr	interval between successive transfers to fresh medium (subculturings)

Genera are listed in alphabetical order with names of families in parentheses.

Acanthamoeba (Acanthamoebidae): Cyst-forming. NNA/Ec, tr 1 year, st 6-9. PPG (axenic), tr 8 weeks, st 12-15. Cry. Note: Some strains potentially pathogenic.

Adelphamoeba galeacystis (Vahlkampfiidae): Cyst-forming, but duration or viability uncertain. NNA/Ec, tr 7-9 weeks, st 18-22. Cry.

Amoeba (Amoebidae): No cysts known.

A. proteus: PC 2-3 mm deep in 90 mm petri dish. Replace liquid and feed with washed *Tetrahymena pyriformis* 3 times a week. Tr 3 weeks, st 18-22. Cry; see also Section 3. Surface-dried storage cultures or *A. proteus*.

A. borokensis: As *A. proteus* but feed daily. Keep liquid no more than 3 mm deep. Because of its short generation time, *A. borokensis* is especially suitable for experimental work. To obtain large numbers for such work, the following method is suggested: To a high-walled grass culture dish, diameter approximately 18-20 cm, add an abundant petri dish culture, then PC to a total depth of 2 cm. Feed daily with washed *Tetrahymena*, gradually increasing the quantity of *Tetrahymena* suspension to 50 ml per day as the

amoebae become more abundant. When the depth of the liquid has reached 5 cm, replace it with fresh PC. Keep culture dish covered. Cry.

A. leningradensis: As a *A. proteus*, but st at 15-19. Cry.

Arachnula impatiens (Arachnulidae): Cyst-forming. Cultured in aqueous suspensions of *Cochliobolus sativus* at 18-22°C.

Cashia limacoides (Hartmannellidae): Non-encysting. NNA/Ec. Multiplies slowly. Subculture as suspension in AS, tr 7-8 weeks, st 18-22. Older cultures may provide more abundant inoculum.

Chaos (Amoebidae)

C. carolinense: Cyst-forming but cysts of no value in maintenance. As *A. proteus*.

C. nobile: No cysts known. MP in 50 mm culture dishes. Feed with a few drops of suspension of *Colpidium striatum* 3 times a week, tr monthly, st 18-20. Cultures may last a year.

Dactylamoeba (Paramoebidae): No cysts.

D. bulla: MP in 50 mm culture dishes. Feed with washed *Tetrahymena* 2 or 3 times a week, tr monthly, st 18-20. Cultures may last many months.

D. stella: CPA/Ec or NNA/Ec, tr 3-4 weeks, st 18-22. May also feed on *Rhynchomonas nasuta*.

Dermamoeba granifera (Thecamoebidae): Non-encysting. CPA, feeding on accompanying bacteria and possibly other small organisms, tr monthly as suspension in PJ, st 18-22. Multiplies slowly, populations never large.

Deuteramoeba (Amoebidae)

D. algonquinensis: No cysts known. CPA/ec with *Hartmannella vermiformis*, tr 2 weeks, st 18-22. Also PJ/r/*Chilomonas*, usually not as good as on CPA.

D. mycophaga: Cyst-forming, but durability of cysts not known, PJ/r/*Chilomonas*, tr 2 weeks, st 18-22.

Didascalus thorntoni (Vahlkampfiidae): Cyst-forming, NNA/Ec, st 18-22. Optimal transfer interval and durability of cysts not known.

Echinamoeba (Echinamoebidae)

E. exundans: Cyst-forming. NNA/Ec, tr 1 year, st 6-9.

E. silvestris: Forms cysts poorly after first few weeks of isolation. NNA/Ec, tr 7-8 weeks, st 18-22.

Entamoebe moshkovskii (Entamoebidae): Subculture in Jones's medium under anaerobic conditions. Cry.

Flabellula spp. (Flabellulidae): All marine, non-encysting. MY75SA with accompanying bacteria, tr 8 weeks or more often depending on vigour of strain, st 18-22. Cry.

Glaeseria mira (Hartmannellidae): Cyst-forming but may lose ability to encyst after years in culture. NNA/Ec. If encysting well, tr 6 months, st 6-9. If not encysting well, tr 3-4 weeks, st 18-22.

Gruberella flavescens (Gruberellidae): Marine, non-encysting. Erds, tr 10 days, st 18-22. MY75SA overlaid with Erds, tr 10-30 days, st 18-22. Cultures decline rapidly if neglected.

Hartmannella (Hartmannellidae)

H. abertawensis: Marine, non-encysting. MY75SA, tr 3-4 weeks as suspension in 75% seawater, st 18-22.

H. cantabrigiensis: Cyst-forming but may suffer reduction in ability to encyst after years in culture. NNA/Ec. If encysting well, tr 6 months-1 year, st 6-9. If not encysting well, tr 3-4 weeks, st 18-22.

H. vermiformis: Cyst-forming. NNA/Ec, tr 1 year, st 6-9.

Heteramoeba clara (Vahlkampfiidae): Marine, brackish, cyst-forming. MY75SA/Ec, tr 7-8 weeks, st 18-22.

Leptomyxa reticulata (Leptomyxidae): Cyst-forming, though encystment ability is reduced after years in culture CPA/Ec or NNA/Ec,

tr 3-4 weeks, st 18-22. Addition of *Vexillifera bacillipedes* as a food organism may improve the cultures.

Mayorella (Paramoebidae)

M. viridis: No cysts known. NNA overlaid with PJ, tr monthly but cultures may be good for more than a year if not allowed to dry out. Keep at 13-15 at all times with normal period of daylight. Contain zoochlorellae, but source of nutrition not adequately investigated.

Other freshwater species: No cysts known. CP/PJ, tr 4 weeks, st 18-22. These species feed on accompanying organisms but the best food source is not known, and cultures should be kept in a normal day/night photoperiod until food has been determined.

Marine species: C75S, or Erds, or MY75SA overlaid with Erds, tr 4 weeks, st 18-22. Feeding on accompanying organisms; keep in normal day/night photoperiod until photosynthetic organisms ruled out as food source. *Cyclidium* accompanying amoebae appears good food source for some strains.

Naegleria (Vahlkampfiidae)

N. gruberi: Cyst-forming. NNA/Ec, tr 1 year, st 6-9. Cry.

N. fowleri: Cyst-forming. NNA/Ec, te 3 months, incubate at 37, st 6-9. SCGYEM (axenic), tr monthly, incubate at 37, st 15-22. Cry. Note: *lethal pathogen.*

Neoparamoeba spp. (Vexilliferidae): Marine, non-encysting. MY75SA, tr 3-8 weeks depending on strain, st 18-22. Cry (*Paramoeba* of Table 2).

Nolandella hibernica (incertae sedis): Marine, non-encysting. MY75SA, tr 7-8 weeks, st 18-22.

Nuclearia (Nucleariidae)

N. delicatula: No cysts known. PJ with *Phormidium faveolarum* fragmented by 30 seconds' exposure to ultrasound, tr 1 week, st 18-20.

N. moebiusi: No cysts known. PJ with addition of a little cooked egg yolk or dried baker's yeast, tr 1 week, st 18-22.

N. radians: No cysts known. Culture as *N. delicatula*.

N. simplex: Cyst-forming. NNA/Ec, tr 7-8 weeks, st 18-22.

Paraflabellula reniformis (Flabellulidae): Marine, possibly brackish, non-encysting. MY75SA, tr 3-4 weeks, st 18-22. For isolates from different parts of the Baltic, try agar of different salinities corresponding to natural source.

Paramoebe eilhardi (Paramoebidae): Marine, non-encysting. Erds with *Amphiprora hyalina*, tr 8 weeks, incubate and st 13-22, with normal day/night photoperiod MY75SA overlaid with Erds, apparently feeding on accompanying bacteria, tr 4-8 weeks, st 18-22, not necessarily in light.

Paratetramitus jugosus (Vahlkampfiidae): Cyst-forming. NNA/Ec, tr 1 year, st 6-9. Euryhaline; strains from saline habitats on MY75SA/Ec, tr 2-3 months, st 18-22. Cry.

Pelomyxs palustris (Pelomyxidae): Freshwater, microaerobic. Not yet brought into proper cultures. Keep collected material in laboratory under conditions maintaining low oxygen tension.

Phreatamoeba balamuthi (incertae sedis): Cyst-forming, but viability of cyst unknown. Jones's medium in tubes, with accompanying bacteria, tr 1 month, st 18-22. Anaerobic; amoebae near bottom of tube.

Platyamoeba (Vannellidae)

P. placida: Cyst-forming. NNA/Ec, tr 6 months, st 6 - 9.

P. stenopodia: Cyst-forming, but ability to encyst reduced or lost after months or years in culture NNA/Ec. If encysting well tr 6 months, st 6-9. If not encysting well, tr 3-4 weeks, st 18-22.

Marine species: Not cyst-forming. MY75SA, tr 4-8 weeks depending on strain, st 18-22. Strains which have declined improve when agar is streaked with *E. coli*.

Polychaos (Amoebidae)

P. dubium: Possibly cyst-forming, but cysts of no value in culturing. Can be maintained as *A. proteus*, or washed *Colpidium striatum* can be substituted for *Tetrahymena* as the food organism.

P. fasciculatum: No cysts known. MP/r/*Chilomonas*, tr 4 weeks, st 18-22. CPA/Ec with accompanying small amoebae, tr 4 weeks, st 18-22. Cultures do not always grow well and need careful attention.

Protacanthamoeba caledonica (Acanthamoebidae): Cyst-forming. NNA/Ec, tr 6 months, st 6-9. Multiplies and encysts more slowly than most strains of *Acanthamoeba*.

Pseudoparamoeba pagei (Vexilliferidae): Marine, non-encysting. MY75S, tr 7-8 weeks, st 18-22.

Pseudothecamoeba proteoides (Thecamoebidae): No cysts known. CP/*Chilomonas*, tr 2-3 weeks, st 18-22. Needs careful attention.

Rhizamoeba (Leptomyxidae)

R. australiensis: Cyst-forming. CPA/Ec or NNA/Ec, tr 3-4 weeks, st 18-22. When strains are young and vigorous they may survive 6 months on slopes at 6-9°.

R. polyura: Marine, non-encysting. MY100SA or C100SA, tr 2 weeks, st 18-22. Needs close attention.

R. saxonica: Marine, non-encysting. MY75SA, tr 3-4 weeks, st 18-22.

Saccamoeba (Hartmannellidae)

S. limax: Non-encysting. NNA/Ec, tr 3-4 weeks, st 18-22. Can also be grown in PJ/r, with added *E. coli* or without added *E. coli* but feeding on accompanying bacteria, tr 2-3 weeks, st 18-22. Cultures in liquid do not become as abundant as those on agar.

S. stagnicola: Cyst-forming but ability to encyst may be reduced or lost after years in culture. NNA/Ec. If encysting well, tr 6 months, st 6-9. If not encysting well, tr 4 weeks, st 18-22.

Sappinia diploidea (Thecamoebidae): Forms sexual cysts, but mixture of cysts and active amoebae should be used to subculture. NNA streaked with abundant *E. coli*, tr 2-3 weeks, st 18-22. Strains appear to last not more than 2 years in clonal culture.

Stachyamoeba lipophora (Gruberellidae): Cyst-forming. NNA/Ec, tr 1 year, incubate 2-3 weeks at 18-22, st 6-9.

Tetramitus rostratus (Vahlkampfiidae): Cyst-forming. NNA/Ec, tr 1 year, st 6-9.

Thecamoeba (Thecamoebidae)

T. quadrilineata: No cysts known. CPA with accompanying bacteria and *Vexillifera bacillipedes*, tr 3-4 weeks, st 18-22. Can also be grown in CP.

T. similis: No cysts known. CPA with accompanying bacteria and sometimes small amoebae, tr 3-4 weeks, st 18-22.

T. sphaeronucleolus: No cysts known. CPA, possibly feeding on small *Vannella* if this is present, tr 3-4 weeks, st 18-22.

T. striata: No cysts known. CPA with accompanying bacteria and *Naegleria gruberi*, which *T. striata* ingests as both amoebae and cysts; tr 3-4 weeks, st 18-22.

T. terricola: No cysts known. CPA with accompanying bacteria and *Acanthamoeba polyphaga*, which *T. terricola* ingests as both amoebae and cysts; tr 7-8 weeks, st 18-22.

Trichamoeba sinuosa (Amoebidae): Cyst-forming but durability of cysts not known. CP/PJ/r/*Chilomonas*, tr 10-14 days, st 18-22. Needs careful attention.

Vahlkampfia (Vahlkampfiidae)

Freshwater species: All cyst-forming, but capacity to form cysts may be reduced after years in culture. NNA/Ec. If encysting well, tr 1 year, st 6-9. If not encysting well, tr 4-8 weeks, st 18-22. The species *V. enterica* grows well at 37° as well as at 18-22°, and cultures should be incubated at both temperatures. Cry.

Marine species: Non-encysting. MY75SA, tr 2-4 weeks, st 18-22.

Vampyrellidium perforans (Nucleariidae): No cysts known. In water with *Chlamydomonas cribrum* either living or heat-killed cells; tr 1 week, st 18-22.

Vannella (Vannellidae): No cysts known.

Freshwater species: NNA/Ec, tr 3-4 weeks, st 18-22.

Marine species: MY75SA, tr 3-4 weeks, st 18-22.

Vexillifera (Vexilliferidae): Non-encysting.

V. bacillipedes: NNA/Ec, tr 2-4 weeks st 18-22. Requires abundant supply of bacteria.

Marine species: MY75SA, tr 3-8 weeks, st 18-22.

Willaertia magna (Vahlkampfiidae): Cyst-forming. NNA/Ec, tr 8-12 weeks, st 18-22. Possibly the cysts will remain viable longer than 12 weeks and could be stored at 6-9° for a long period, but this possibility needs to be tested. Some strains have been maintained axenically in SCGYEM.

2. CRYOPRESERVATION

Freshwater Protozoa in the vegetative stage of the life-cycle are very sensitive to freezing. Successful cryopreservation methods have been described from some species of naked amoebae (Tab. 1). Most of these methods were developed at the American Type Culture Collection (ATCC).

2.1. PRESERVATION PROCEDURE

Usually the cells to be frozen are grown in the medium and under the conditions of feeding, light and temperature recommended for their normal maintenance. Then vegetative material is harvested in the mid- to late log phase of growth. Organisms which produce cysts are allowed to reach the point of maximum encystment. Generally the minimum number of cells required for successful preservation is a final concentration of 10^6/ml. Cells are usually concentrated by centrifugation. The final cell preparation is dispensed in 0.5 ml aliquots into the appropriate ampoule for freezing. Two types of ampoules are used: plastic (20 ml screw-cap polypropylene cryales with round bottom) and glass. A larger, soft-glass shell vial (14.2 mm · 85.0 mm) is used as a container for the inner smaller vial. The outer

vials are prepared in advance by covering the bottom with silica gel granules. These outer containers are heated at 100 °C over night. The silica gel granules should be dark blue in colour after heating. They are the moisture indicator during storage. Smaller vials are washed, plugged with absorbent cotton wool, sterilised, labelled and resterilised for use as the "inner" vials which will contain the cell suspension.

In accordance with the methods used at the ATCC, the most suitable cryoprotective agent for cryopreservation of protozoa is dimethyl sulphoxide (DMSO). Unless otherwise indicated the cryoprotectant is prepared in doubled to desired final concentration. The Seitz filter-sterilised stock solution of DMSO (100%) (cannot be membrane-filtered) should be diluted to 5% final concentration in the cell suspension to be frozen. The time between exposure of the cells to the cryopro-tective agent and subsequent freezing (equilibration time) is carefully controlled. For protozoan material this time is limited to 15-40 minutes.

For controlled freezing of protozoa an automatic programmable freezing unit (Cryo-Med, Division of Weld Metal, Inc.) with two thermocouple probes, one in the chamber and one in the ampoule containing the cell preparation, is used. This unit permits variation of the rates of cooling.

Uncontrolled freezing is accomplished by placing ampoules into a wire rack and putting the rack on the bottom of a mechanical refrigerator at -55° to -60°C for one hour. Then the ampoules are placed into a liquid nitrogen refrigerator.

All cultures frozen in liquid nitrogen are thawed rapidly in a 35°C water bath until the last trace of ice is dissipated (standard thawing method). From 40-60 seconds is needed for glass ampoules, with agitation, while 60-120 seconds is needed for plastic ampoules. The culture samples are transferred aseptically to an appropriate growth medium immediately after thawing. If the medium is broth, 5-6 ml per tubes are used.

2.2. RECOVERY

Each strain is cultured in the proper growth medium to insure that its ability to be propagated in culture and its growth characteristics have not been altered by the freeze preservation procedure (Tab. 2). Viability is established by three successive subcultures from the frozen sample. The percentage of recovery is based upon counts of pre- and post-freeze cells. Motile cells from

Table 2

Thawing, recovery	Applicable to:
Standard	*Acanthamoeba astronyxis, A. comandoni*
Standard	*Acanthamoeba*, Adelphamoeba, Naegleria, Vahlkampfia*
Standard**	*Acanthamoeba*, Flabellula, Lingulamoeba, Paramoeba, Paratetramitus*
Rehydration: Add 0.5 ml of medium to shell vial and incubate at 25 °C in the dark	*Paratetramitus, Acanthamoeba, Naegleria, Vahlkampfia**
Standard	*Entamoeba**
Ultrarapid thawing at 37 °C	*Amoeba* spp.

* Methods are applicable to all members of the genus listed unless a specific method is cited for a strain or species
**Entire contents of ampoule are spread uniformly over agar plate

the final pre-freeze preparation are counted using a haemocytometer; this constitutes the pre-freeze count. One hour after thawing, motile cells from an aliquot of the thawed cells are counted, using a haemocytometer; this constitutes the post-freeze count. Not all protozoans are motile an hour after thawing. The percentage is determined by dividing the post-freeze count by the pre-freeze count, e. g., $1 \cdot 10^6$ cells post-freeze / $2 \cdot 10^6$ cells pre-freeze $\cdot$ 100 = 50% recovery. As a rule, a flourishing culture is established from several cells recovered by the freezing-thawing procedure. So standard recovery means that more than 1% of the cells survived after freezing.

3. SURFACE-DRIED STORAGE CULTURES OF A. PROTEUS

Some amoeboid protozoans encyst to protect themselves from harmful environmental conditions. These species can be stored by inducing encystment under appropriate conditions and storing the resistant cysts. However, *Amoeba* spp. do not encyst. Japanese scientists (Ueda & Ogawa 1978) developed a simple method for storage of *A. proteus* at 4°C (in the refrigerator) or at room temperature. The steps of this procedure are as follows:

1) A plastic box or petri dish is filled to a depth of 2-3 mm with 3% agar.

2) 5 ml of a thick *Amoeba* culture in Chalkley's medium is cultured for several weeks by the addition of

pasteurised rice grains after the agar has cooled. The liquid medium is spread over the agar surface by gentle agitation.

3) The open box is placed in darkness at room temperature for approximately 15 hours. In 15 hours the amoebae became spherical.

4) The box is sealed with vinyl tape and stored at 4-10°C.

5) To recover active organisms from the surface-dried storage culture the agar squares should be immersed in Chalkley's medium in a petri dish. Within 10 minutes normal amoebae with cytoplasmic streaming will be recovered. According to these authors 90% of the cells survived after storage for one year at 4°C and 60% after storage for the same time at room temperature.

NOTE: Recently it was shown that storage of both mitotic and interphase *A. proteus* at 4°C can result in hereditary changes (Afon'kin & Kalinina 1987).

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Wichterman R. (1986) The Biology of Paramecium. 2 ed. Plenum Press, New York.

Articles from books:

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Line drawings should be made suitable for reproduction in the form of well-defined line drawings and should have a white background. Computer printouts of laser printer quality may be accepted. Avoid fine stippling or shading. Line drawings must be no more than twice final size.

Photographs

Photographs at final size should be sharp, with a glossy finish, bromide prints, and mounted on firm board. Photographs grouped as plates must be trimmed at right angles very accurately mounted with edges touching. The engraver will then cut a fine line of separation between figures. Magnification should be indicated.

Proof sheets and offprints

Authors will receive one set of page proofs for correction and return to the Editor. Fifty reprints will be furnished free of charge.

IN PREPARATION:

V. S. Golemansky and **A. I. Darwish**: The coccidian parasites (Apicomlexa: Coccidia) of the herbivorous mole-rat *Spalax* (*Microspalax*) *ehrenbergi Nehring* (Rodentia: Spalacidae) from Syria - **M. A. Jareño**: Macronuclear behavior during development of doubletsin *Onychodromus acuminatus* (Ciliophora, Hypotricha) - **G. Esteban, C. Tellez** and **L. M. Bautista**: The indicator value of *Tetrahymena thermophila* populations in the activated-sludge process - **H. -P. Vornlocher** and **D. -P. Häder**: Biochemical and spectroscopic investigation of the presumed photoreceptor for phototaxis in Amoebae of the cellular slime mold, *Distyostelium discoideum*.

SUBSCRIPTION and BACK VOLUMES

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