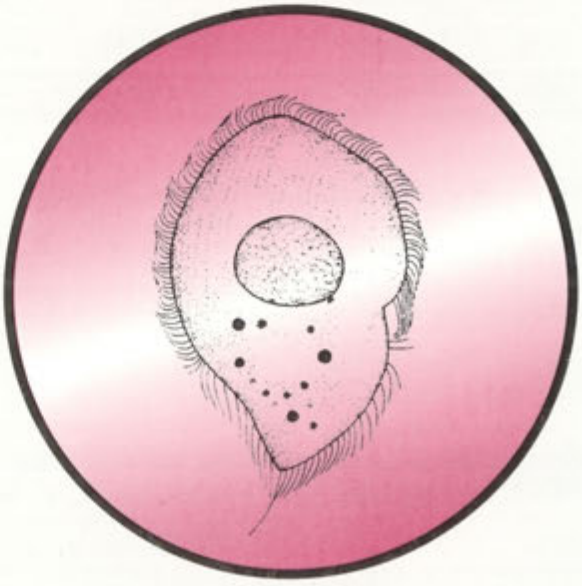
ACTA PROTOZOOLOGICA



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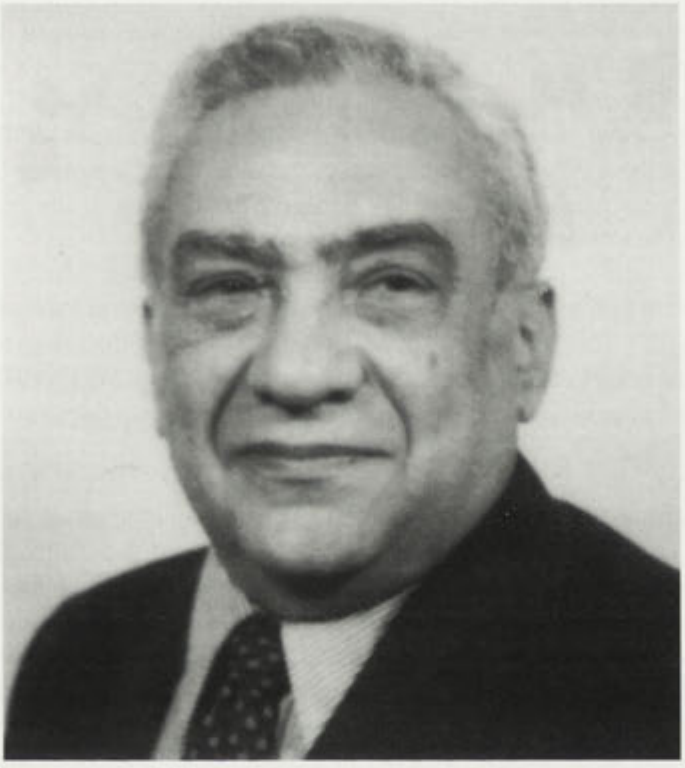

BRONISŁAW M. HONIGBERG

(1920-1992)

On the first of May, 1992, Professor Dr Bronisław M. Honigberg left us for ever. He went - as a result of heart disease, two weeks before his seventy-second birthday. He left numerous friends all over the world, many scientific problems solved and a many to be undertaken by his students. We all will remember him as a great scientist and as a man, and we are with his wife Rohoda and sons Paul Mark and Martin Philip.

Professor Dr Bronisław Honigberg was born in Warsaw, Poland, on 14th May, 1920. The vicissitudes of the II World War forced him to leave country. He settled in the United States, where he found his second motherland. He conducted his postgraduate studies in zoology at the University of California in Berkeley. He specialized in protozoology and parasitology under guidance of the prominent protozoologist Prof.Dr H.Kirby, getting his Ph.D. in 1950. Then he moved to the Massachusetts University at Amherst, where he obtained his successive academic degrees. From 1961 he held the post of professor while from 1980 he was director of the Parasitological Center in this University. Professor Honigberg worked as visiting professor at several universities, including: Columbia University (NY), John Hopkins University (MP), Harvard University (MA), the National Institute of Health at Bethesda, the University of Edinburgh at Great Britain and International Laboratory for Research on Animal Diseases, Nairobi, Kenya.

Most of Professor Honigberg's scientific studies are devoted to parasitic flagellates *Trichomonadida* and *Kinetoplastida*. They concern their structure, cytology and later ultrastructure, various aspects of their physiology, biochemistry, immunology and pathogenicity. This studies have contributed a great deal to our knowledge of those groups of flagellates, particularly *Trichomonadida*. A significance of a great many of these papers, because of their synthetic character, overstep beyond the groups described. Professor Honigberg was also widely known expert in the systematics of Protozoa. Under his guidance the Committee on Taxonomy and Taxonomical Problems of the Society of Protozoologists worked out and published a modified Systematics of Protozoa in 1964. Professor Honigberg participated also in the work in perfecting that systematics in the Committee of Systematics and Evolution of the Society of Protozoologists publishing "A newly revised classification of the Protozoa" in 1980. Both those papers have had a considerable influence on present-day protozoology.

Professor Honigberg took active part in the international scientific life, actively participating in numerous congresses and symposia, including almost all the International Congresses on Protozoology. He presented papers requested by the organizers at following International Congresses on Protozoology: 3rd Congress at Leningrad (1969), 4th Congress at Clermont-Ferrand (1974), 5th Congress in New York (1977). At the 6th International Congress on Protozoology in Warsaw (1981) he organized a special session to discuss recent proposals on Protozoan systematics.

Professor Honigberg's teaching activity was extremely wide. He lectured in zoology, protozoology and parasitology, and conducted courses on immunity to animal parasites and the physiology of parasites. He launched 21 doctors, many of whom are now professors at various universities in the United States of America and other countries. At the Massachusetts University at Amherst many protozoologysts and parasitologists from United States and other countries went through long-therm training, gaining valuable experience in research.

Professor Honigberg was also involved in editorial work. He was a member of Editorial Board of the "Journal of Protozoology" from 1959 and for nine years he held the post of its Editor-in-Chief. He was also a member of the editorial boards of several well-known journals publishing articles on protozoology, including the "Transactions of the American Microscopical Society", the "Journal of Parasitology" and the "Zeitschrift für Parasitenkunde". Shortly ago he was appointed to the Editorial Board of **"Acta Protozoologica"**.

Professor Honigberg was a member of the Academy of Sciences of New York and a member of several scientific societies among which should be mentioned the Society of Protozoologists (honorary membership), the American Society of Parasitologists,the American Society of Zoologists, the Royal Society of Tropical Medicine and Hygiene, the American Microscopical Society, the Society of Systematic Zoology, and the American Association for the Advancement of Science. In two societies, the Society of Protozoologists and the American Microscopical Society, Professor Honigberg had the function of president.

Since 1965 Professor Honigberg was the initiator and member of International Commission on Protozoology.

Professor Honigberg was an outstanding protozoologist and parasitologist of world importance, extremely active in the scientific life. He maintained friendly relations with numerous scientists all over the world, always ready to offer help and advice.

Stanisław Kazubski
Leszek Kuźnicki
Jerzy Sikora

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The Indicator Value of *Tetrahymena thermophila* Populations in the Activated-sludge Process

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Summary. The presence of the ciliate *Tetrahymena thermophila* was studied in relation with the efficiency of the disposal process in an activated-sludge sewage treatment plant. Stepwise Discriminant Analysis (Dixon, 1989) was applied to physico-chemical and biological variables in order to classify days with good or regular efficiency in the disposal process according to the abundance of *T. thermophila* in aeration tanks. The results indicate that the ciliate *T. thermophila* may be a useful organism in the prediction of plant performance.

Key words. *Tetrahymena thermophila*, activated sludge, bioindicator.

INTRODUCTION

Activated-sludge processes are environments characterized by the presence of high organic matter concentrations and high bacterial cell densities. Large populations of ciliates therefore grow in sewage-treatment plants (Curds 1975, 1982; Curds and Cockburn 1970a; Curds et al. 1968; Madoni and Ghetti 1981; Esteban et al. 1991a,b). During biological treatment of waste water different species of ciliates are developed and they are useful organisms in the understanding of the disposal process. The presence of different ciliate species may indicate differences in the stage of the disposal process or differences in the effluent quality (Curds 1975, Curds and Cockburn 1970b, Esteban 1991a). Therefore, ciliates can be used to predict environmental characteristics. *Tetrahymena thermophila* is a ciliate species commonly found in activated-sludge sewage-treatment processes and little is known about its role in these artificial ecosystems.

T. thermophila is characterized by growing upon bacteria and suspended particles and has the capacity of removing soluble organic substances from the medium by diffusion through the cell membrane (Hill 1972, Holz 1973). This capacity has let to cultivation of *Tetrahymena spp.* in organic solutions in the absence of bacteria (Curds and Cockburn 1970b). This feeding behavior has also let this ciliate grow under certain conditions in the ecosystems where it lives - e.g. with extreme organic matter concentrations, like activated sludge (Esteban et al. 1991a,b).

The aim of the present study is to explain the indicator value of *T. thermophila* during biological treatment of waste water in an activated-sludge sewage-treatment plant, and its role in the prediction of plant performance.

Paper presented on the 2nd International Conference of Hungary on Protozoology "Current Problems in Protozoan Ecology",26-30 August 1991, Tihany, Hungary.

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Table 1.

Physico-chemical variables measured in the sewage treatment plant

I. VARIABLES OF THE QUALITY OF WASTE WATER ENTERING THE AERATION TANKS
Chemical oxygen
Biological oxygen
Suspended solids
Dissolved solids
Conductivity
Water temperature
II. VARIABLES OF THE EFFLUENT
Chemical oxygen
Biological oxygen
Color
Conductivity
pH
III.OPERATIONAL VARIABLES
Retention time
Sewage flow
Average dissolved oxygen
Organic load
Volumetric load
Mixed liquor suspended solids
Sludge volume (in 30 minutes)
Mohlman volumetric index

MATERIALS AND METHODS

Daily counts of ciliated protozoa were made at the treatment plant within a few minutes of collection. The technique of Madoni (1984, 1988) was used: five replica of 0.05 ml sub-samples of activated sludge were counted. In each sample ciliates were identified to species level (Esteban et al. 1991a).

Physico-chemical variables of the sewage-treatment plant were measured at the same time following Standard Methods (1989) (Table 1).

For all multivariate analyses a data matrix with the results of biological and physico-chemical measurements was drawn up. The normality assumption was checked for each variable distribution and appropriate transformation were made [variable= ln (variable + 1)].

Stepwise Discriminant Analysis (Dixon 1989) was used to classify days according to the process efficiency through the number of ciliates present. The data of days with good disposal process (days with sufficient number/ml of *T. thermophila*) and regular disposal process (low number/ml of *T. thermophila*) were subjected to Stepwise Discriminant Analysis. These two groups were made according to the effluent COD value (COD ≤ and > 100 mg/l) in relation to the total number/ml of *T. thermophila* observed in aeration tanks. For testing the accuracy of this statistical analysis a 20% sub-sample of the original data recorded was randomly extracted. Values of the coefficients were recalculated for the remainder 80% of the ciliate population data and the new equation obtained from this discriminant analysis was applied to the sub-sample (20%). Finally, after studying the results from the first two discriminant analyses, a third one was made considering three groups: low, medium, and high density of *T. thermophila* in aeration tanks.

RESULTS

Results from the different discriminant analyses are shown in Fig. 1, and Tables 2-4.

The BOD and conductivity in settled sewage, the temperature in aeration tanks, and the effluent COD are the variables that classify days with low number/ml of *T. thermophila* and days with high density of the ciliate studied. These variables discriminate correctly 80% of the cases following Jackknife classification (Tables 2, 3). Results that are corroborated with those from the test discriminant analysis (Table 2, 3). In the test discriminant analysis (Table 2) there is an input and an output of variables, but those of measures of quantity of organic matter concentration are maintained. This change of variables may be influenced by the reduction of a 20% in the sample size.

Table 4 shows results from the third discriminant analysis considering three groups of efficiency in the disposal process. The plot of discriminant scores showing the three groups are represented in Fig. 1.

DISCUSSION

The results from the statistical analyses applied to the study of the dynamics of *Tetrahymena thermophila* populations in activated-sludge process show that this ciliate may be used to classify the efficiency of the biological treatment.

The close agreement between the results of the discriminant analyses indicates that our conclusions are statistically valid.

Not only do our results show the indicator value of *T. thermophila* populations, but also the direct association between this ciliate with high organic matter concentrations and effluent quality is corroborated. Previous studies refer to the different ecology of this ciliate with respect to other species from the same community in the aeration tanks, possibly due to its relationship with organic matter concentration and to feeding mechanisms (Esteban et al. 1991a,b). *Tetrahymena sp.* can utilize dissolved organic matter directly through the cellular membrane (Hill 1972, Holz 1973). This ability lets *T. thermophila* survive during periods of organic matter stresses and lower levels of bacterial densities, characteristics that do not benefit the development of the rest

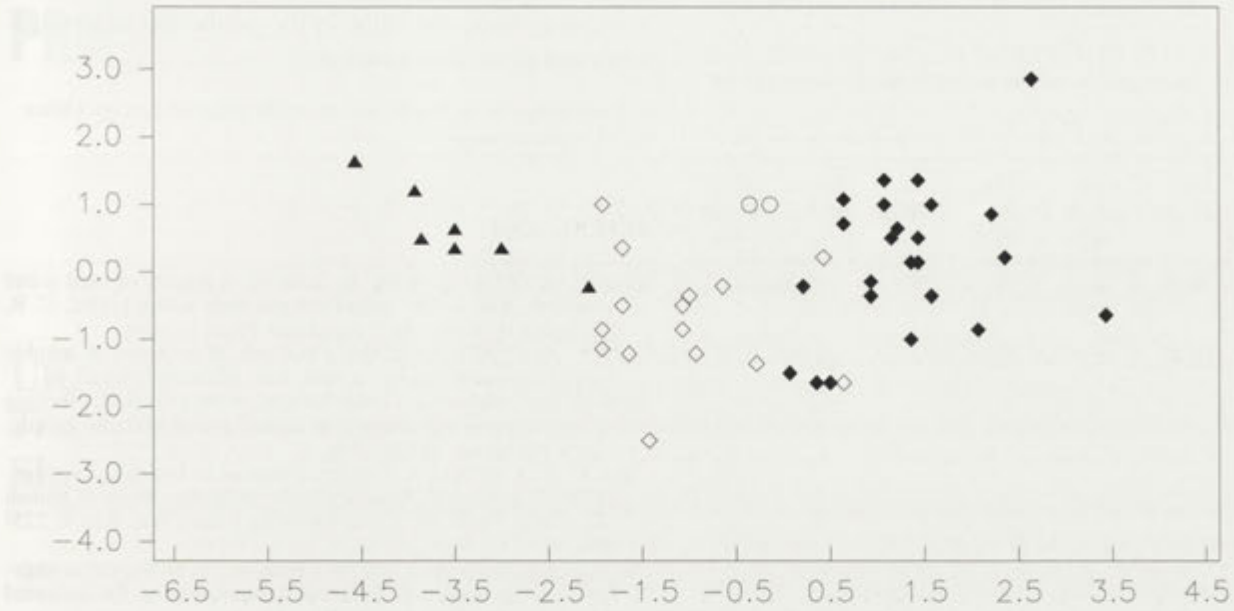

Fig. 1. Plot of discriminant scores of the density of *Tetrahymena thermophila* in aeration tanks. Note the three groups corresponding to bad, regular, and good disposal process. Abbreviations : ▲ good process = high number of ciliates/ml, ◊ regular process = medium number of ciliates/ml, ♦ bad process = low number of ciliates/ml, o two values for the same plot

of ciliates (Esteban et al. 1991a,b). In the present study, variables selected through the different discriminant analyses are those that measure the quantity of organic matter in sewage - BOD and dissolved solids in settled sewage - and COD and color of the effluent. Therefore, our results let us to classify days with good or regular efficiency in the disposal process according to the number/ml of *T. thermophila* observed, when this ciliate grows during biological treatment, and according to the variables aforementioned.

Under high organic matter concentrations the population density of the ciliate community in aeration tanks decreases. Under such conditions *T. thermophila* can not only ingest particles, but also take up organic matter from the medium, and this results in population increase.

Table 2

Selected variables and standardized coefficients based on Stepwise Discriminant Analyses

	100% of the sample		80% of the sample	
VARIABLE	Low nº/ml of *T. thermophila*	High nº/ml of *T. thermophila*	Low nº/ml of *T. thermophila*	High nº/ml of *T. thermophila*
Susp. solids settled sewage	-	-	59.7	63.3
B.O.D. settled sewage	-11.3	-13.9	15.2	18.3
Conductivity settled sewage	304.0	296.8	-	-
Water temper. in aer. tanks	177.7	191.1	142.4	130.7
Effluent COD	4.3	10.7	27.7	22.5
Effluent color	-	-	-5.0	-8.2
Effluent conductivity	-	-	164.2	169.7
CONSTANT	-1255	-1260	-1029	-1032

Table 3

Classification matrix for discriminant analyses. The number of cases classified into groups are shown (Jackknifed classification)

	100% of the sample			80% of the sample		
GROUP	% correct	Low no/ml (n)	High no/ml (n)	% correct	Low no/ml (n)	High no/ml (n)
Low no/ml	80.8	21	5	78.9	15	4
High no/ml	79.2	5	19	76.2	5	16
Test low no/ml	-	-	-	-	6	1
Test high no/ml	-	-	-	-	0	3
TOTAL	80.0	26	24	77.5	26	24

Table 4

Classification matrix for discriminant analyses considering three groups. The number of cases classified into groups are shown (Jackknifed classification)

GROUP	% correct	Low no/ml	Medium no/ml	High no/ml
Low no/ml	76.9	20	6	0
Medium no/ml	70.6	4	12	1
High no/ml	85.7	0	1	6
TOTAL	76.0	24	19	7

During favorable periods for the other ciliate species in the same community – with high population densities –, of *T. thermophila* is capable of feeding on dissolved organic matter, without competing for available food materials. Thus *T. thermophila* maintains its population levels.

This "strategy" lets this ciliate live for long periods in the aeration tanks. As a consequence *T. thermophila* is an efficient organism clarifying effluent and has an interesting indicator value in the prediction of sewage-treatment plant performance.

Acknowledgments. Thanks are due to the Spanish Foreign Office for financial support.

REFERENCES

Curds C. R. (1975) Protozoa. In: Ecological aspects of used-water treatment. Vol. I. The organisms and their ecology, (Ed. C. R. Curds and H. A. Hawkes). Academic Press, London.

Curds C. R. (1982) The ecology and role of protozoa in aerobic sewage treatment processes. Ann. Rev. Microbiol. 36: 27-46.

Curds C. R., Cockburn A. (1968) Studies on the growth and feeding of *Tetrahymena thermophila* in axenic and monoxenic culture. J. gen. Microbiol. 54: 343-358.

Curds C. R., Cockburn A. (1970a). Protozoa in biological sewage-treatment process. I. A survey of the protozoan fauna of British percolating filters and activated-sludge plants. Wat. Res. 4: 225-236.

Curds C. R., Cockburn A. (1970b). Protozoa in biological sewage-treatment processes. II. Protozoa as indicators in the activated sludge process. Wat. Res. 4: 237-249.

Curds C. R., Cockburn A., Vandyke J. M. (1968) An experimental study of the role of the ciliated protozoa in the activated sludge process. Wat. Pollut. Control 67: 312-329.

Dixon W. J. (1989) BMDP Statistical Software. Univ. California Press, Los Angeles.

Esteban G., Téllez C., Bautista L. M. (1991a) Dynamics of ciliated protozoa communities in activated-sludge process. Wat. Res. 25: 967-972.

Esteban G., Téllez C., Bautista L. M. (1991b) Effects of habitat quality on ciliated protozoa communities in sewage treatment plants. Environmental Technology 12: 381-386.

Hill D. L. (1972) The biochemistry and physiology of *Tetrahymena*. Academic Press, New York.

Holz G. G., Jr. (1973) The nutrition of *Tetrahymena*: essential nutrients, feeding, and digestion. In: Biology of *Tetrahymena*, (Ed. A. M. Elliott). Dowden, Hutchinson & Ross, Inc. Stroudsburg, Pennsylvania.

Madoni P. (1984) Estimation of the size of freshwater ciliate populations by a sub-sampling technique. Hydrobiologia 111: 210-216.

Madoni P. (1988) I protozoi ciliati nel controllo di efficienza dei fanghi attivi. Centro Italiano Studi di Biologia Ambientale. Reggio Emilia, Italy.

Madoni P., Ghetti P. F. (1981) The structure of ciliated protozoa communities in biological sewage treatment plants. Hydrobiologia 83: 297-215.

Standard Methods for the Examination of Water and Wastewater. (1989) 17 ed. American Public Health Association, American Water Works Association and Water Pollution Control Federation. Washington, D. C.

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Ultrastructural Peculiarities of the Meiotic Prophase in the Ciliate *Tracheloraphis totevi* (Karyorelictida): Synaptonemal Complexes and Sheaths around Bivalents

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Summary. The fine structure of the micronuclei has been studied in the course of the meiotic prophase during conjugation in *Tracheloraphis totevi*. During pachytene, a marked heterochronism between chromosomes is observed: at the periphery of swollen micronuclei, the bivalents are weakly condensed and display conspicuous synaptonemal complexes. Nearer to the center of the nucleus, the bivalents are already compact and show no SC's. In early diakinesis, all bivalents are alike and display prominent chiasmata. During this time, groups of annular structures measuring 50-100 nm and resembling pore complexes appear in the nuclear matrix. Such free (non membrane bound) pore complexes attach to the bivalents and fuse with each other, forming regions of a continuous finely fibrillar sheath which stand apart from the chromosome body to 20-40 nm. In late diakinesis, each bivalent is entirely surrounded by a about 20 nm thick sheath, except the centromere region. At the place of chiasmata, the sheath is continuous, passing from one homologous chromosome onto the other. The presence of such isolating sheaths around diakinetic chromosomes is possibly related to their transcriptional inactivity during this period and may be analogous to the formation of a karyosphere in the late meiotic prophase during oogenesis in some animals (Insecta, Amphibia).

Key words. *Tracheloraphis totevi*, micronucleus, meiosis, chromosomes, bivalents.

INTRODUCTION

Many ciliates belonging to the order Karyorelictida, especially to the family Trachelocercidae which are typical sand-dwelling (psammophilic) marine forms, have rather large and not very numerous chromosomes in their micronuclei. During conjugation, these micronuclei, as usual, undergo two-division meiosis followed by a postmeiotic mitosis and give rise to pronuclei, which are multiple in all karyorelictids where conjugation has been studied (Raikov 1958, 1963; Dragesco and Raikov 1966; Kovaleva 1972, 1987). The formation of more than one pair of pronuclei in each conjugant has been considered a primitive feature, a rudiment of gametogenesis (Raikov 1972).

At the light microscopical level, bivalents have been observed in the meiotic prophase of the micronuclei of some Trachelocercidae. In *Tracheloraphis phoenicopterus, Trachelocerca coluber, Tracheloraphis caudatus* they tend to have one chiasma and, correspondingly, cruciform shape in diakinesis (Raikov 1958, 1963; Dragesco and Raikov 1966). But in *Trachelonema sulcata* having especially large chromosomes, some bivalents form two or even three chiasmata which gradually terminalize (Kovaleva 1972).

The process of meiosis have never been studied with the electron microscope in any member of the family

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Trachelocercidae. Only in another family of the karyorelictids, the Loxodidae, a study of the early stages of the meiotic prophase of *Loxodes striatus* have revealed synaptonemal complexes between the paired chromosomes and free pore complexes in the nuclear matrix (Bobyleva 1984). A fragmentary study of conjugation in some Geleiidae has also shown the presence of synaptonemal complexes in the micronuclei during the meiotic prophase in *Geleia orbis* (Nouzarède 1976).

The present paper describes the fine structure of synaptonemal complexes, free pore complexes, and peculiar sheaths around diplotene bivalents during the meiotic prophase in a trachelocercid, *Tracheloraphis totevi*. This species is a typical representative of the psammophilic ciliate fauna of the Black sea (Kovaleva and Golemansky 1979). The fine structure of the multiple pronuclei of this species and the mechanism of their transfer into the other conjugant have been described earlier (Kovaleva 1987). Also the ultrastructural modifications of the paradiploid macronuclei of *T.totevi* have been investigated (Kovaleva and Raikov 1988).

A part of the results included in the present paper has been published in Russian (Kovaleva and Raikov 1990).

MATERIAL AND METHODS

All available material was a natural one; attempts to obtain conjugation in the laboratory with living specimens previously collected were unsuccessful. The material has been collected at the beaches of the Black Sea, mainly at the Bulgarian coast (Kovaleva and Golemanski 1979). The ciliates were extracted from samples of the superficial layers of sandy sediments taken at water depths of less than 1 m; conjugating pairs were among vegetative individuals. The material was fixed with a freshly prepared mixture (1:1) of 4% glutaraldehyde in 0.05 M cacodylate buffer with 0.003 M $CaCl_2$ (pH 7.4) and 2% OsO_4 in the same buffer, with the addition of 7.5% saccharose to the mixture. The ciliates were fixed for 20 min at 20°C in darkness. After a wash with the same buffer, at first with and then without saccharose, the conjugants were pre-embedded one-by-one in 2% agar gel and stored in 70% alcohol until a final embedding in epon-araldite. The sections were stained with uranyl acetate and lead citrate and studied with JEM-100C, JEM-7A and Tesla BS-500 electron microscopes.

Abbreviations

CB – condense chromosomal bivalent
CC – condensed chromosome
CH – chiasma
CR – centromere region
DB – decondensed bivalents
G – granules associated with pachytene bivalents
Ma – macronucleus
Mi – meiotic micronucleus
NE – nuclear envelope
PC – pore complexes
S – sheath around bivalents
SC – synaptonemal complex

RESULTS

The nuclear apparatus of vegetative individuals of *Tracheloraphis totevi* is represented by a single compact group of nuclei, where the macronuclei, counting 16-22, are peripheral while the two micronuclei occupy the center of the complex (Kovaleva and Golemansky 1979). Vegetative micronuclei are oval, filled with condensed chromatin, and measure about 3 µm in length. During conjugation, the partners unite with their non-ciliated longitudinal stripes; there is no cytoplasmic fusion but only interdigitation of the pellicles of the two conjugants ensuring their tight union (Kovaleva 1987).

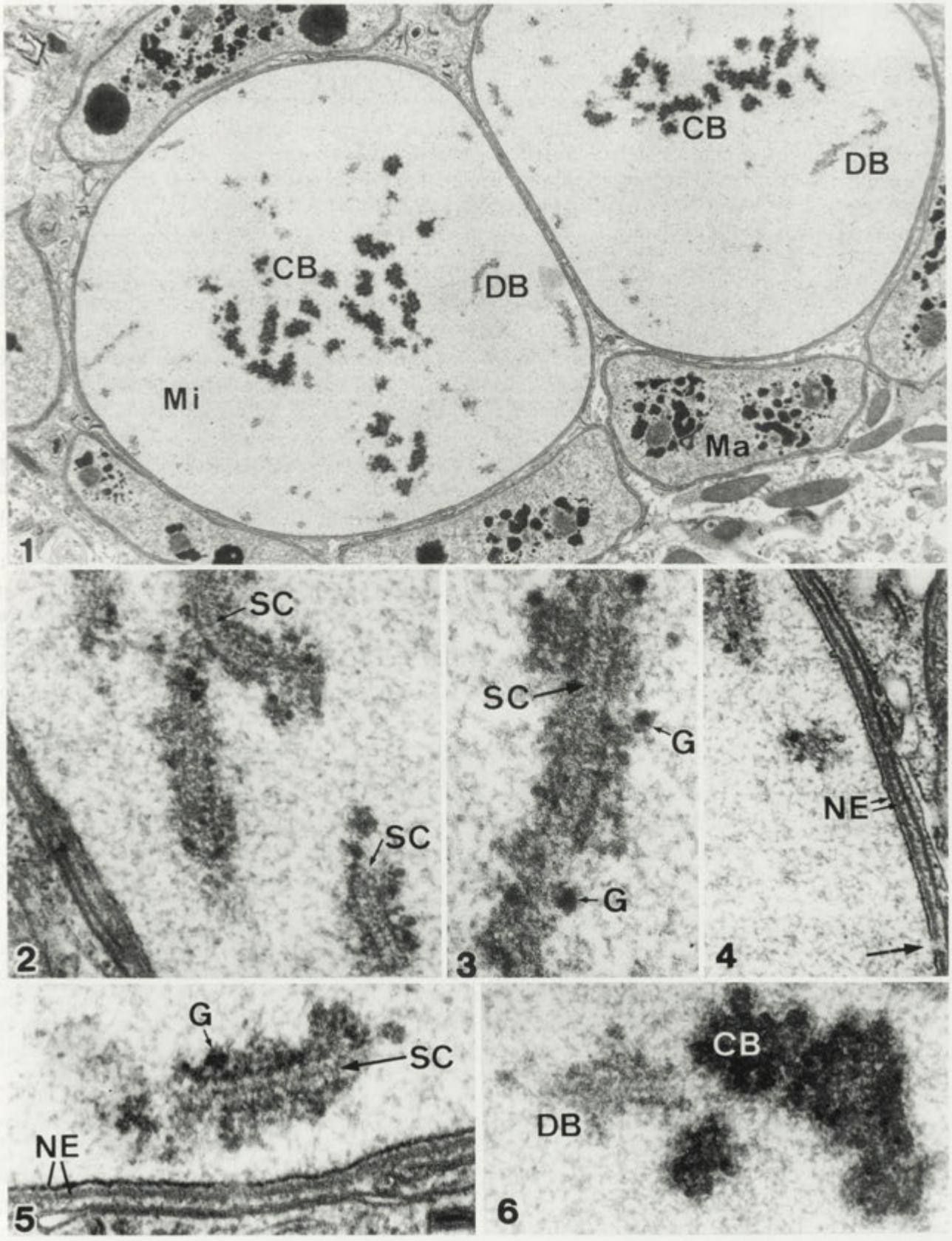
Following the union of the conjugants, both micronuclei of each cell enter the meiotic prophase; they conspicuously swell, reaching 11-12 µm in diameter, and become larger than the surrounding macronuclei (Fig. 1). Two stages of the meiotic prophase, late pachytene and diakinesis, occurred most frequently in the material available and were investigated in more detail.

The late pachytene stage

At the pachytene stage, the micronuclei look like large electron-lucent spheres occupying the core of the nuclear group (Fig. 1). The group itself loosens and gaps appear between previously adjacent macronuclei.

The meiotic micronuclei show a conspicuous dimorphism of the aspect of the chromosomes: in the central region of each nucleus, the chromosomes (or their parts) are electron dense and compact, whereas in the peripheral zones of the same nucleus, they are thin,

Figs. 1-6. Pachytene stage of the meiotic prophase. 1- General aspect of the two meiotic micronuclei (Mi) encircled by numerous macronuclei (Ma) and containing both condensed (CB) and decondensed bivalents (DB) (x 7,500). 2- Synaptonemal complexes (SC) at the periphery of a micronucleus (x 62,000). 3- A synaptonemal complex showing dense granules (G) outside the lateral elements (x 83,000). 4- The nuclear envelope (NE) showing rare pores (at arrow) and collapsed cisternae outside the nucleus (x 49,000). 5- Asynaptonemal complex (SC) near the nuclear envelope (NE) showing an electron dense inner membrane (x 83,000). 6- A decondensed bivalent (DB), containing a synaptonemal complex, near a condensed bivalent (CB) (x 62,000)



much less dense (Fig. 1), and show synaptonemal complexes along them, indicating that the chromosomes are in reality bivalents. The structure of the synaptonemal complexes is typical (Figs. 2-5): in favorably oriented sections, they are seen to consist of two dense lateral elements, a synaptic space about 50 nm wide, and a central or axial element, 10-12 nm in diameter, with transverse filaments connecting it to the lateral elements (Figs. 2, 3, 5). The outer sides of the lateral elements are typically studded with large electron dense granules measuring 30-50 nm in diameter (Figs. 3, 5). Likely enough, the compact and the decondensed aspects of the chromosomes correspond to different parts of the same chromosomes along their length. In the compact regions, no synaptonemal complexes could be discerned (Fig. 6). Probably these regions correspond to early diplotene when chromosomes condense, homologs partly disunite, and synaptonemal complexes disappear. If so, we may speak of heterochronism (heterocyclism) between different chromosome regions, the peripheral ones being still in pachytene. The difference between the two is shown in Fig. 6.

The nuclear envelope of the micronuclei remains continuous, though it seems to be distended. The pore complexes in it become very scarce (Figs. 4, 5). The inner membrane of the envelope usually looks more electron dense than the outer one. On the outer side of the envelope, there are usually 1-2 additional layers of collapsed agranular cytoplasmic cisternae (Figs. 4, 5).

The diakinesis stage

During diakinesis, all chromosomal bivalents look alike, irrespective of their position in the meiotic micronuclei (no heterocyclism is observed any more). The micronuclei have approximately the same size as in pachytene (about 12 μm), and the nuclear group becomes even looser, the gaps between the surrounding macronuclei increasing (Fig. 7). The nuclear envelope of the meiotic micronuclei remains continuous, showing rare pore complexes in it.

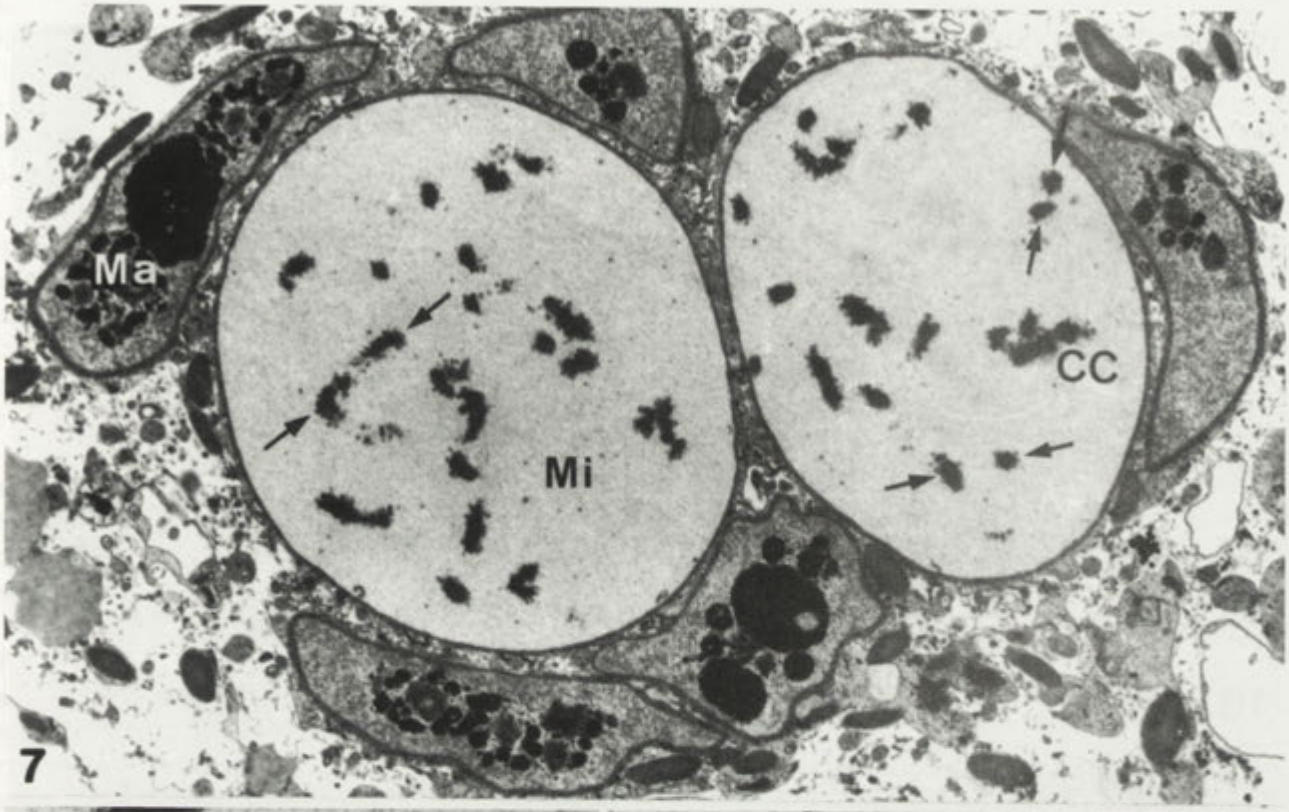
In early diakinesis, the chromosomes that have united into bivalents look thick and electron-dense and are scattered throughout the micronucleus (Fig. 7). They are about 0.2 μm thick. In most cases, the profiles of chromosomes are single and thus seem to correspond to chromosome regions disunited from their homologues distal to or between the chiasmata (Fig. 8). However, even in that case most profiles of the chromosomes appear to be paired in survey micrographs (Fig. 7). Less frequently, the section passes through or near a chiasma (Fig. 9). The presence of two chromatids in each homologue is not evident at the electron microscopical level (Figs. 7-10). No synaptonemal complexes can now be revealed any more.

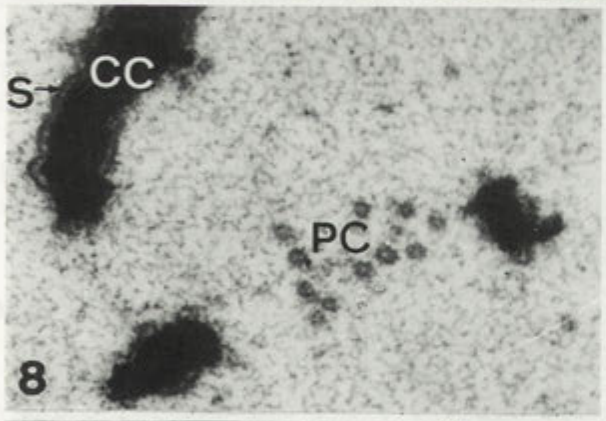
During diplotene, ring-like (annular) structures measuring 500-100 nm in diameter and resembling pore complexes begin to appear in the nuclear matrix. However, they are not bound to any membranes and occur in groups inside the meiotic micronucleus, usually in the vicinity of some bivalents (Figs. 8, 9). The annular structures differ from typical pore complexes of the nuclear envelope by the significant variability of their size. Some ring-like structures show, besides the dense annulus, also a central element in form of a granule (Figs. 9, 10).

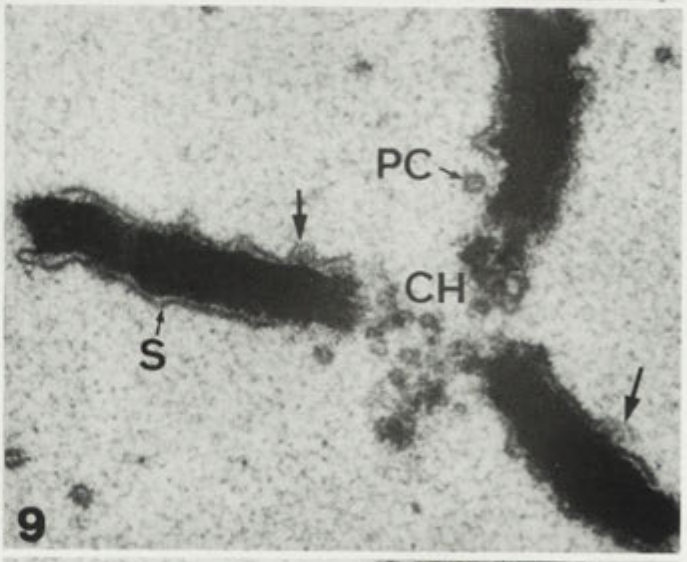
Simultaneously, portions of lamellae parallel to the surface of the chromosome begin to appear during early diakinesis (Fig. 8). These lamellae are about 20 nm thick and are separated from the chromosome proper by a clearance of about 30-40 nm. In early diakinesis, the lamellae cover only some regions of the homologous chromosomes (Fig. 8). In mid-diakinesis, the lamellae increase in surface area, and they tend to form a continuous sheath around the chromosomes (Figs. 9-10). It can be observed that many annular structures (free pore complexes) fuse with the lamellae or with the chromosome sheath (Figs. 9, 10, at arrows). It can be supposed that the sheath is formed by fusion of the material of the free pore complexes on the surface of the bivalents.

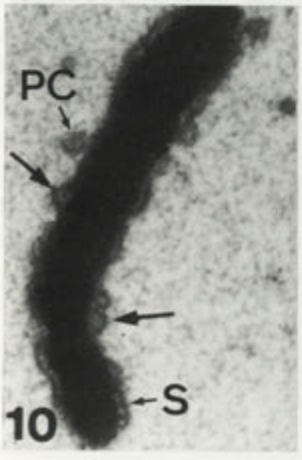
At later stages of diakinesis, no free pore complexes can be observed in the micronucleus any more (Figs. 11 - 14). The sheaths around the chromosome bivalents are now almost complete (Figs. 11 - 13). One may see in grazing sections of the sheath that it has a

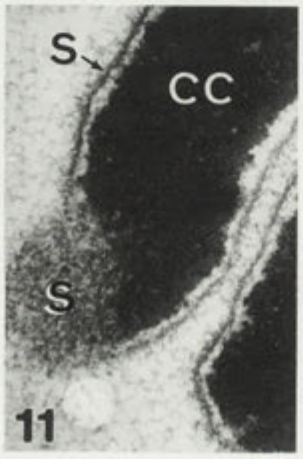
Figs. 7-12. Early and middle diakinesis stages. 7- Two meiotic micronuclei (Mi) in early diakinesis, showing condensed chromosomes (CC) which appear as pairs mostly (opposite arrows) (x 6,000). 8- Structures resembling free pore complexes (PC) between the chromosomes (CC) which begin to form sheaths (S) (x 35,000). 9- Meiotic bivalent cut near a chiasma (CH) and showing free annuli resembling pore complexes (PC) and their fusion (at arrow) with the forming sheaths (S) (x 35,000). 10- A chromosome arm with free annuli resembling pore complexes (PC) and those fusing (at arrow) with the sheaths (S) (x 35,000). 11- Chromosome arm (CC) showing the sheaths (S) in grazing and normal sections (x 45,000). 12- Detail of a chiasma region (CH) on a condensed bivalent, showing details of the sheaths (S) (x 60,000)

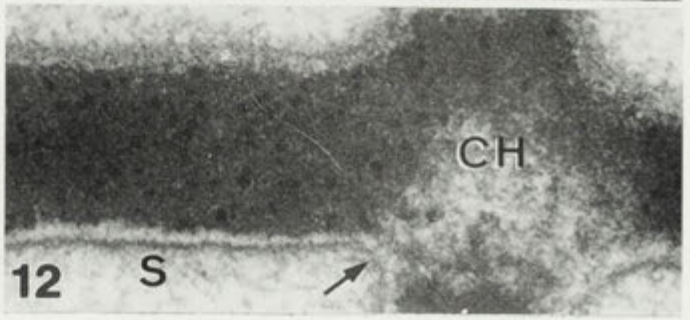


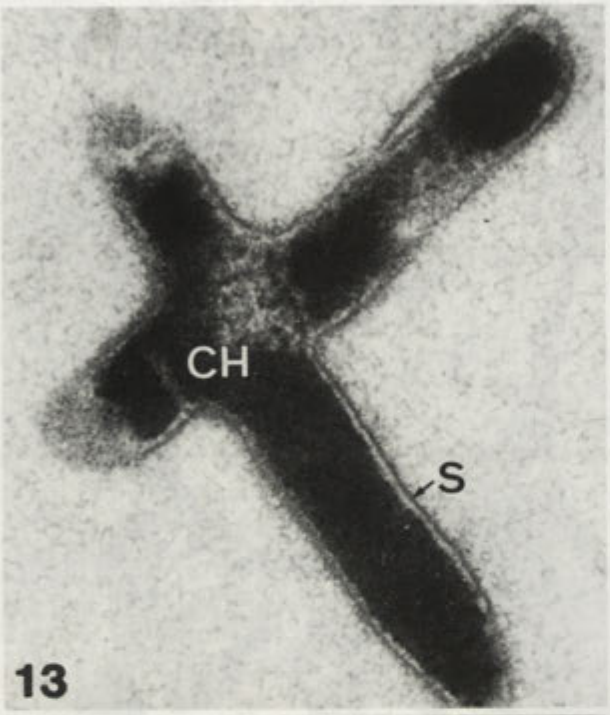




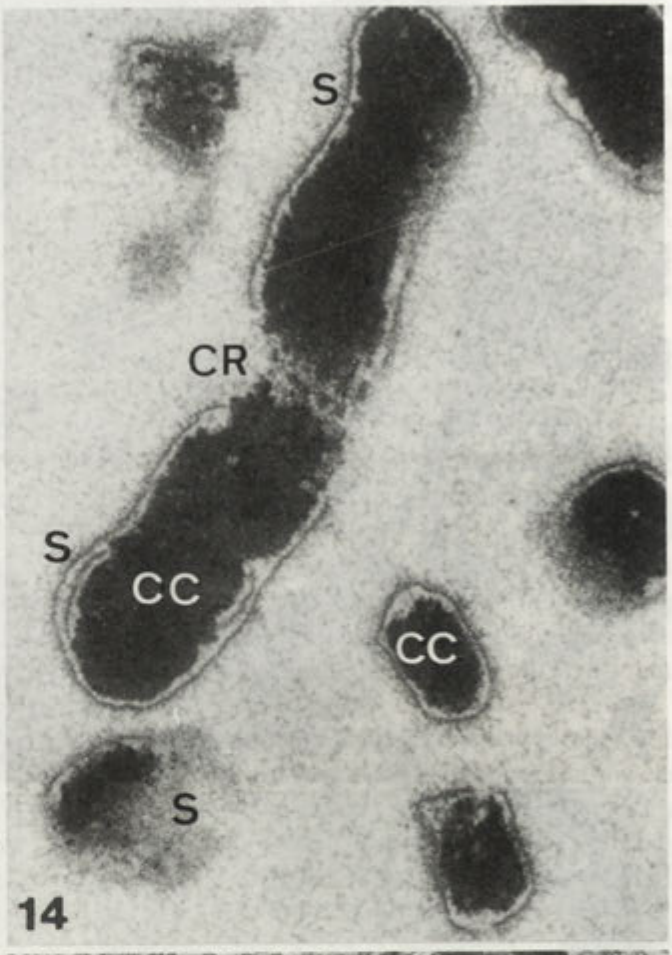








13



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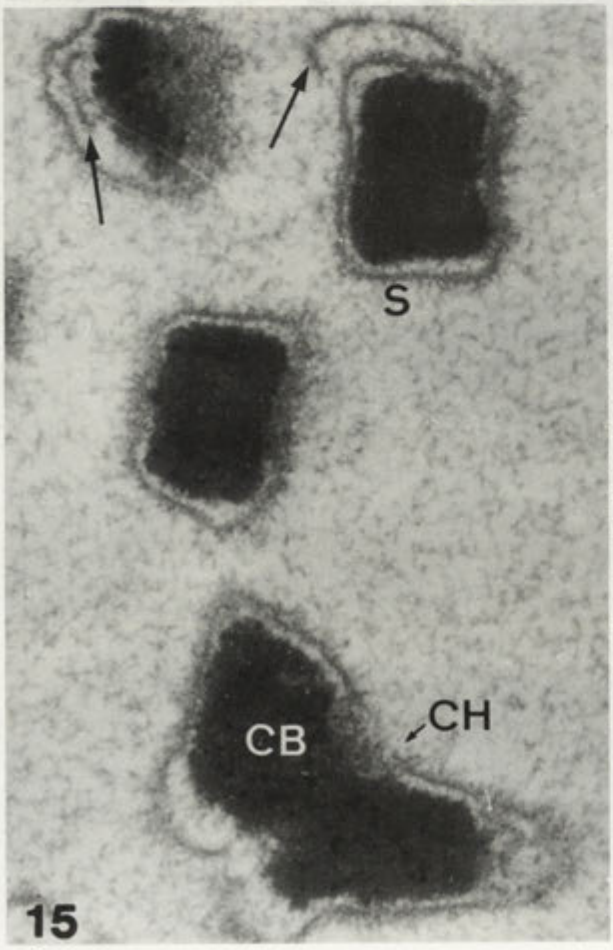

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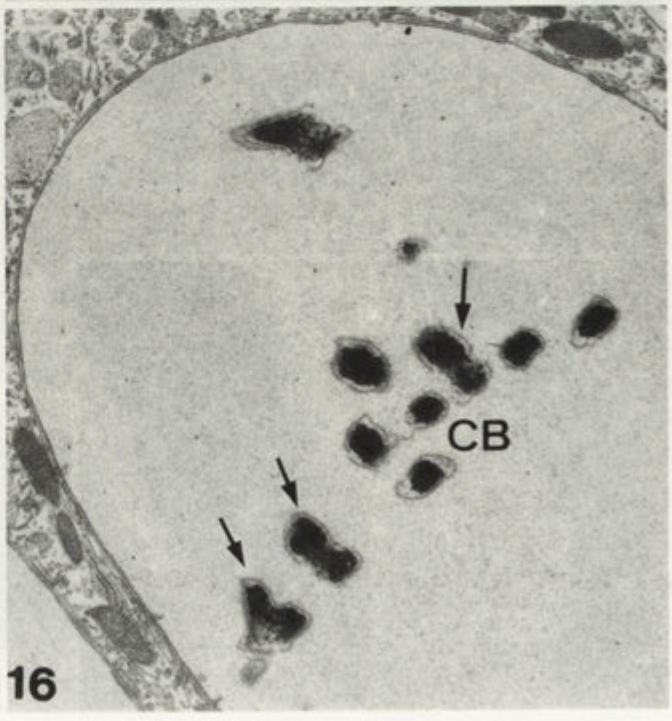

16

finely fibrillar texture (Fig. 11, 14, 15). Its component fibrils are approximately 5-6 nm thick (Figs. 11, 12). The thickness of the lamella forming the sheath is about 20 nm, as before (Figs. 11, 12). In the regions of chiasmata, the sheath extends from one homologous chromosome onto the other without any gap (Figs. 12, 13).

The sheath is clearly interrupted in the centromere region only, which is distinguished by its less thickness and electron density, i.e. in the region of the primary constriction of the chromosome (Fig. 14). In some places, the sheaths become double, small areas of similar lamellae lying either beneath the main sheath (Fig. 14) or outside it (Fig. 15).

On transverse sections of the chromosomes, the sheath is practically always continuous (Figs. 14, 15, 16). This means that the sheaths cover not only the poleward sides of the homologous chromosomes but their lateral sides too.

In late diakinesis, the chromosomes condense even stronger (Figs. 14-16), and the bivalents tend to approach the equatorial region of the micronucleus (Fig. 16). The sections of some chromosomes look dumbbell-shaped or bipartite which probably means that the respective sections met a chiasma (Figs. 15, 16), The nuclear envelope of the micronuclei remains intact and continuous; the number of pore complexes in the envelope does not exceed 20 per 1 μm^2 which is approximately 3 times less than the pore frequency in the envelopes of vegetative micronuclei. Even in late diakinesis, the micronuclei still lack any spindle microtubules.

DISCUSSION

Synaptonemal complexes have so far been observed in meiotic micronuclei of quite a limited number of ciliates: *Paramecium* (Stevenson 1972), *Blepharisma* (Jenkins 1973), *Dileptus* (Vinnikova 1976), *Didinium* (Karadzhan 1977), *Stentor* (Skarlato 1982). The only karyorelictean known to possess this structure is *Loxodes striatus* (Bobyleva 1984). As in *T.totevi*, the synaptonemal complexes in *Loxodes* connect rather strongly decondensed homologous chromosomes and show a well expressed central element and a structured synaptic space. However, the chromosomes of *Loxodes* do not show any heterocyclosis observed in *T.totevi*: the aspect of chromosome profiles is similar regardless of their position in the meiotic micronucleus during either zygotene or pachytene. The dense granules on the lateral elements of the synaptonemal complexes, which are conspicuous in *T.totevi*, are not so evident in *Loxodes* (Bobyleva 1984).

Loxodes striatus is also the only ciliate where free annular structures have been observed inside meiotic micronuclei (Bobyleva 1984). This peculiarity may thus turn out to be a feature of meiosis proper to the karyorelictids, but further investigations are needed to check this supposition. In *Loxodes*, the annular structures appear earlier than in *T. totevi*: they are present already in zygotene and they form a large cluster in pachytene. However, formation of any kind of sheaths around chromosomal bivalents, with or without participation of the free annular structures, has not been demonstrated in *Loxodes*, possibly because the diakinesis stage is hardly distinguishable in this ciliate. The functional role of the very conspicuous free intranuclear annular structures in *Loxodes* thus remains unknown.

Besides *Loxodes*, free intranuclear annuli resembling pore complexes have been observed in a variety of cells. They have been found to participate, along with the protein components of synaptonemal complexes, in the formation of the "capsule" of the karyosphere enclosing the chromosomal set in oocytes of some insects and amphibians (Fill and Moens 1973, Gruzova and Parfenov 1977, Parfenov 1979, Filatova et al. 1982). Free pore complexes sometimes occur in somatic cell nuclei as well (Troitskaya et al. 1981).

The fine structure of the chromosomal bivalents at the end of the meiotic prophase (in diplotene or diakinesis) has been investigated in not many ciliates: *Dileptus* (Vinnikova 1976), *Didinium* (Karadzhan 1977), *Paramecium* (Stevenson 1972), *Stentor* (Scarlato 1982), and *Ephelota* (Grell and Meister 1984). Despite the differences in the degree of chromosome condensation during this stage (weak in *Paramecium*, moderate in *Dileptus* and *Didinium*, and strong in *Stentor* and *Ephelota*), no fibrous or other isolating sheaths around

Figs. 13-16. Middle and late diakinesis stages. 13- A bivalent with a chiasma (CH) completely surrounded by the sheath (S) (x 24,000). 14- The centromere region (CR) of a homologous chromosome where the sheath (S) is interrupted; transverse sections of other chromosomes (CC) (x 45,000). 15- Transverse or oblique sections through chromosomes (CC) with sheaths (S) which frequently show additional lamellae (at arrows); one bivalent is cut through a chiasma (CH) (x 65,000). 16- Late diakinesis showing bivalents (CB), some at or near a chiasma (at arrows) (x 15,000)

mitotic bivalents have been observed in any of these ciliates. In a few other protozoans where late stages of the meiotic prophase were studied with the electron microscope, similar sheaths have not been found (Mignot 1980). Apparently, such sheaths are novel in protists.

Among multicellular animals, non-membranous envelopes isolating single bivalents or groups of bivalents have been reported to exist during oogenesis of the gall-flies, Cecidomyiidae (Kunz et al. 1970; Gruzova et al. 1987). Their relation to pore complexes and/or synaptonemal complexes, though not excluded, has not been established.

The question about the nature and the possible functions of the sheaths in *T. totevi* cannot presently be answered unambiguously but deserves some discussion.

First, the sheaths show some resemblance to the "diffuse" kinetochores of the holocentric chromosomes of some animals and plants, e.g., to those occurring during meiosis and mitosis in the nematodes *Ascaris* and *Parascaris* (Goldstein 1978, Goday et al. 1985), during mitosis only in the bugs *Rhodnius* and *Oncopeltus* (Buck 1967, Comings and Okada 1971), during mitosis in the plant *Luzula* (Bokhari and Godward 1980) and in some algae (Godward 1985). However, three arguments can be raised against homologization of the sheaths with large kinetochore lamellae: (a) The "diffuse" kinetochores typically cover only the poleward surfaces of the chromosomes, as shown in *Oncopeltus, Rhodnius, Luzula*, whereas the sheaths cover the bivalents of *Tracheloraphis totevi* from all sides. (b) The kinetochores, also those of holocentric chromosomes, are usually trilaminar (review: Godward 1985), which is not the case of the sheaths in *T. totevi*. (c) The sheath in *T. totevi* is interrupted in the region of the primary constriction of the chromosome, i.e. where the kinetochore should in any case be present. A study of later stages of meiosis, when microtubules would become attached to the chromosomes, is necessary to finally solve this question.

Second, the sheaths around meiotic bivalents in *T. totevi* somewhat resemble two-membraned envelopes around separate mitotic chromosomes, occurring in the gregarine *Stylocephalus* (Desportes 1970). In telophase, these bimembrane envelopes fuse and become transformed into the common nuclear envelope of the daughter nucleus. A similar process occurs in mitosis of another gregarine, *Grebnickiella* (Molon-Noblot and Desportes 1980). However, in meiosis of the latter species there are apparently no membranous envelopes around the bivalents (Molon-Noblot and Desportes 1977). However, the sheaths around the bivalents in *Tracheloraphis totevi* are definitely non-membranous.

Third, the fibrillar sheaths around the bivalents in *T.totevi* are to a certain extent analogous to the wall of the karyosphere, though being externally not similar to it. The karyosphere is a complex of tightly packed bivalents, isolated by a non-membranous wall or capsule, which occurs in the late meiotic prophase of the oocytes of some animals: insects, amphibians and others (review: Gruzova 1975). As already mentioned, multiple free intranuclear pore complexes and/or synaptonemal complexes take part in the formation of the karyosphere capsule in both insects and amphibians (Fiil and Moens 1973, Gruzova and Parfenov 1977, Parfenov 1979, Parfenov and Gruzova 1984, etc.). It may consequently be supposed that the fibrous sheaths around the bivalents in *Tracheloraphis* and the karyosphere around the inactive set of bivalents in various animals are built of components of pore and synaptonemal complexes. It is possible that sheaths around separate bivalents in Cecidomyiidae have the same origin (Gruzova et al. 1985).

Apparently, also functional analogy can be drawn between the fibrous sheaths and the karyosphere. In both cases, we may speak of isolating non-membranous envelopes around inactivated chromosomes during late meiotic prophase. The difference is, however, that the karyosphere isolates the whole chromosome set together from the rest of the karyolymph, while the sheaths isolate each bivalent separately.

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REFERENCES

Bobyleva N.N. (1984) (Synaptonemal complexes and intranuclear pore complexes in the meiotic prophase of the ciliate *Loxodes striatus*). Cytologiya (Leningrad) 26: 138-142 (in Russian, English summary).

Bokhari F.S., Godward M.B.E. (1980) The ultrastructure of the diffuse kinetochore in *Luzula nivea*. Chromosoma 79: 125-136.

Buck R.C. (1967) Mitosis and meiosis in *Rhodnius prolixus*: The fine structure of the spindle and diffuse kinetochore. J. Ultrastr. Res. 18: 489-501.

Comings D.E., Okada T.A. (1971) Holocentric chromosomes in *Oncopeltus*: kinetochore plates are present in mitosis but absent in meiosis. Chromosoma 37: 177-192.

Desportes I. (1970) Ultrastructure des Grégarines du genre Stylocephalus: la phase enkystée. Ann. Sci. Nat. Zool. 12e sér., 12: 73-169.

Dragesco J., Raikov I. (1966) L'appareil nucléaire, la division et quelques stades de la conjugaison de *Tracheloraphis margaritatus* (Kahl) et *T.caudatus* sp.nov. (Ciliata, Holotricha). Arch. Protistenk. 109: 99-113.

Fiil A., Moens P.B. (1973) The development, structure, and function of modified synaptonemal complexes in mosquito oocytes. Chromosoma 41: 37-62.

Filatova L.S., Stolyarov S.D., Zbarsky I.B. (1982) (Ultrastructure of the karyosphere and nuclear envelope in oocytes of the grass frog, *Rana temporaria*, after treatment with nucleases and extraction of chromatin and alcali-soluble proteins). Cytologiya (Leningrad) 24: 131-136 (in Russian, English summary).

Goday C., Ciofi-Luzzato A., Pimpinelli S. (1985) Centromere ultrastructure in germ-line chromosomes of *Parascaris*. Chromosoma 91: 121-125.

Godward M.B.E. (1985) The kinetochore. Int. Rev. Cytol. 94: 77-105.

Goldstein P. (1978) Ultrastructural analysis of sex determination in *Ascaris lumbricoides* var. *suum*. Chromosoma 66: 59-69.

Grell K.G., Meister A. (1984) Beitrage zur Ultrastruktur der Konjugation von *Ephelota gemmipara* R.Hertwig (Suctoria). Protistologica 20: 65-86.

Gruzova M.N. (1975) The karyosphere in oogenesis. Cytologiya (Leningrad) 17: 219-237 (in Russian, English summary).

Gruzova M.N., Batalova F.M., Kozlova-Yermolajeva L.V. (1987) (Oogenesis in the predatory gall-fly, *Aphidoletes aphidimyza*. I. Ultrastructure of young oocytes in the pupae and imago). Cytologiya (Leningrad) 29: 251-259 (in Russian, English summary).

Gruzova M.N., Parfenov V.N. (1977) Ultrastructure of late oocyte nuclei of *Rana temporaria*. J. Cell Sci. 28: 1-13.

Jenkins R.A. (1973) Fine structure. In: *Blepharisma*. The biology of a light sensitive protozoan (Ed. A. C. Giese). Stanford Univ. Press, Stanford, Calif. 39-93.

Karadzhan B.P. (1977) (Ultrastructure of the micronucleus of *Didinium nasutum* (Ciliophora, Gymnostomata) during meiosis). Cytologiya (Leningrad) 19: 1327-1332 (in Russian, English summary).

Kovaleva V.G. (1972) Meiosis and some stages of conjugation in the holotrichous ciliate *Trachelonema sulcata*. Protistologica 8: 83-90.

Kovaleva V.G. (1987) The pronuclei of the lower ciliate *Tracheloraphis totevi* (Karyorelictida). Arch. Protistenk. 134: 367-377.

Kovaleva V.G., Golemansky V.G. (1979) (Psammobiotic ciliates of the Bulgarian coast of the Black sea). Acta Protozool. 18: 265-284 (in Russian, English summary).

Kovaleva V.G., Raikov I.B. (1988) Peculiarities of the ultrastructure of paradiploid macronuclei during conjugation in the lower ciliate *Tracheloraphis totevi*. Arch. Protistenk. 136: 305-315.

Kovaleva V.G., Raikov I.B. (1990) (Fibrous sheaths around meiotic bivalents in the lower ciliate *Tracheloraphis totevi*). Cytologiya (Leningrad) 32: 572-577 (in Russian, English summary).

Kunz W., Trepte H.H., Bier K. (1970) On the function of the germline chromosomes in the oogenesis of *Wachtliella persicariae* (Cecidomyiidae). Chromosoma 30: 180-192.

Mignot J.-P. (1980) Étude ultrastructurale de la pédogamie chez *Actinophrys sol* (Héliozoaire). II. Les divisions de maturation. Protistologica 16: 205-225.

Molon-Noblot S., Desportes I. (1977) Mise en évidence des complexes synaptonématiques dans le noyau méiotique d'un sporozoaire, la grégarine *Grebnickiella gracilis* (Grebnicki), parasite de la scolopendre *Scolopendra cingulata*. C.R. Acad. Sci. Paris 285 (D): 217-219.

Molon-Noblot S., Desportes I. (1980) Étude ultrastructurale des mitoses gamogoniques de la grégarine *Grebnickiella gracilis* Gr., parasite de la scolopendre *Scolopendra cingulata* L. Considérations sur les mitoses schizogoniques des sporozoaires (Apicomplexa). Protistologica 16: 395-411.

Nouzarède M. (1976) Cytologie fonctionnelle et morphologie experimentale de quelques protozoaires ciliés mésopsammiques géants de la famille des Geleiidae (Kahl). Bull. Station Biol. Arcachon n.sér. 28: 1-315.

Parfenov V.N. (1979) The karyosphere during late oogenesis in *Rana ridibunda*. Europ. J. Cell Biol. 19: 102-108.

Parfenov V.M., Gruzova M.N. (1984) (Peculiarities of formation of the karyosphere in *Rana ridibunda*. Electron microscopical data). Cytologiya (Leningrad) 26: 165-168 (in Russian, English summary).

Raikov I.B.(1958) Der Formwechsel des Kernapparates einiger niederer Ciliaten. I. Die Gattung *Trachelocerca*. Arch. Protistenk. 103: 129-192.

Raikov I.B. (1963) Some stages of conjugation of the holotrichous ciliate *Trachelocerca coluber*. Cytologiya (Leningrad) 5: 685-689 (in Russian, English summary).

Raikov I.B. (1972) Nuclear phenomena during conjugation and autogamy in ciliates. In: Research in Protozoology (Ed. T. T. Chen). Oxford-New York: Pergamon Press 4: 147-289.

Skarlato S.O. (1982) Electron microscope study of the micronuclei of the ciliate *Stentor coeruleus* during meiosis. Protistologica 18: 281-288.

Stevenson I. (1972) Ultrastructure of nuclear division in Paramecium aurelia. III. Meiosis in the micronucleus during conjugation. Austral. J. Biol. Sci. 25: 775-799.

Troitskaya L.P., Kuzmina S.N., Buldyaeva T.V., Zbarsky I.B. (1981) Electron microscopic characterization of the nuclear matrix and of its fractions. Cytologiya (Leningrad) 23: 620-625 (in Russian, English summary).

Vinnikova N.V. (1976) Conjugation in the ciliate *Dileptus anser*. I. Ultrastructure of micronuclei during mitosis and meiosis. Protistologica 12: 7-24.

Zatsepina O.V., Poljakov V.Yu., Chentsov Yu.S. (1982) (Formation of the nuclear envelope around metaphase chromosomes under the effect of hypotonicity). Cytologiya (Leningrad) 24: 5-10 (in Russian, English summary).

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Macronuclear Behavior During Development of Doublets in *Onychodromus acuminatus* (Ciliophora, Hypotrichia)

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Summary. Doublets of *Onychodromus acuminatus* develop from cysts of two cells encysted together (Jareño 1977). This kind of cyst is composed of plasma membrane, common cytoplasm and two sets of nuclei and is protected by a well-developed cyst wall. During excystment this spherical and undifferentiated cell is transformed into a complex and polarized organism that has a binary axis of symmetry. The two macronuclei stretch forming sharp points that reach the cortex. Two primordia of ciliature are organized early and two synchronized morphogenetical processes lead to formation of the doublet. When cortical morphogenesis is almost finished the points of the macronuclei retract.The changes of the macronuclei throughout the development of the doublets have been studied. This paper describes, for the first time in ciliates, the behavior of the double set of macronuclei at both light microscope, and ultrastructural levels and shows that these events are correlated with the formation of bundles of fibers and the development of cortical morphogenesis of doublets.

Key words. *Onychodromus acuminatus*, doublets, development, macronuclear behavior.

INTRODUCTION

Double forms in ciliates (doublets) consist of two cells joined in different positions (homopolar, heteropolar, mirror image) and variable degrees of union. Doublets have been known for a long time to appear spontaneously in cultures, without authors commenting about their origin. Doublets can also be produced by stopping division or conjugation by shock treatments, including different physico-chemical factors (Calkins 1925, Fauré-Fremiet 1948, Frankel 1962, Grimes 1973, 1982, Grimes and Hammersmith 1980, Jerka-Dziadosz 1983, Nanney et al. 1975, Shi and Frankel 1990, Totwen-Nowakowska 1965). The homopolar doublets of *Onychodromus acuminatus* develop naturally after a joint encystment (Jareño 1977) of two cells.

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This hypotrichous ciliate has an asymmetrical shape with well developed cortical organelles for food ingestion (adoral zone of membranelles, undulating membrane) and motility (fronto-ventral and transverse cirri). It has also two rows of marginal cirri. All these organelles, and even the infraciliature, disappear during encystment. Two sets of ciliature are newly developed during excystment of a doublet. The macronuclear events at both light microscopy and the ultrastructural levels are analyzed here, and it is shown how the cortical pattern and the shape of the doublet are constructed in relation to positioning fibers during this developmental process.

MATERIALS AND METHODS

The strain of *Onychodromus acuminatus* utilized, was isolated from a pond in the National Reservation of Doñana (Huelva, Spain). The ciliates were maintained at 21°C in soil medium and fed with

Chlorogonium sp. The latter is cultivated under axenical conditions. The ciliates encyst when the food is exhausted and excyst when soil medium and food are added. Two kind of cysts were formed. Cysts with one macronucleus, formed by one ciliate encysted and cysts with two macronuclei, formed by two ciliates encysted together.

The simplified protargol (silver proteinate) technique used consists of impregnating the cysts in accord with Tuffrau (1976) and afterwards treating the cysts with a low speed developer (Jareño 1984). The spherical form of the cyst is almost retained after protargol impregnation. This is an advantage because the structures maintain their relative positions, but can not be focused in the same micrograph.

For ultrastructural studies, the cysts were picked up at various intervals of time after the start of the excystment and fixed for 30 min. in a solution of 2% glutaraldehyde in phosphate buffer pH 7.4, mixed (v/v) with a solution of 2% osmium tetroxide in distilled water. The cysts were postfixed in the same osmium solution for 30 min., washed in phosphate buffer, and embedded in Araldite. The fixation was carried out at laboratory temperature. The sections were stained with uranyl acetate (6 min.) and lead citrate (15 min.).

RESULTS

The resting cysts from which doublets arise were formed by joint encystment of two cells of *O. acuminatus*.

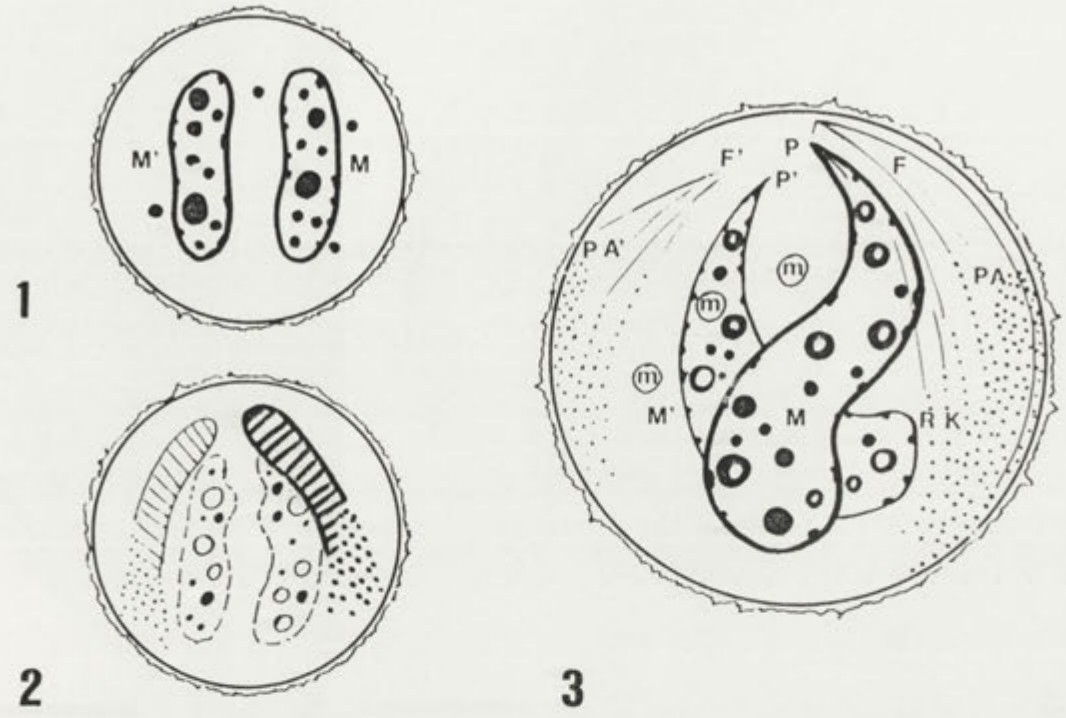
The cyst develops in aproximately two hours and a half.

The formation of the double cyst will not be described here as it has been reported elsewhere (Jareño 1977), but for better understanding of the morphogenetic process, the cyst from which the doublet arises must be briefly described here. This kind of cyst consist of two cells encysted together that have lost their entire ciliature and infraciliature during encystment. It has three or four micronuclei (on which we will not comment because changes were not observed during early excystment) and two macronuclei, each formed by the union of the four macronuclei typical of this species. They are in a common cytoplasm surrounded by a plasma membrane. All this is protected by a well developed cyst wall that, at ultrastrucutral level, has three conspicuous layers (ectocyst, mesocyst and endocyst) along with an inner granular layer. Thus the cyst is an undifferentiated cell with a double nuclear equipment, protected by a cyst wall (Fig. 1). At ultrastructural level no separation is observed between the two components inside the cyst. Only the nuclei maintain their individuality.

The development of the macronuclei during excystment can be summarized in three figures: At the beginning of this process (Fig. 1) and at the end (Fig. 2) the macronuclei have a similar appearance at the light microscopic level. They have the form of lentils and show rounded edges. Between these two stages, however, significant morphological and ultrastructural modifications occur in the macronuclei. Early in excystment the macronuclei begin to stretch, forming sharp points that reach the cortex. In Figs. 3 and 4, the macronuclei are shown in their most elongated appearance. When the macronuclei contact the cortex, bundles of structures that appear as fibers under the light microscope are observed (Figs. 3-10).

In resting cysts and during the first moments of excystment no kinetosomes have been observed. Afterwards, kinetosomes are seen in both spatial and temporal relation to the fibers (Figs. 3, 5, 7, 8, 10). Fig. 3 is a semischematic drawing from micrographs at different optical sections of an excysting doublet. The pointed ends of the macronuclei (P, P') are observed. The bundle of fibers (F) beginning near the P point runs along the spherical surface of the cyst. This bundle can also be observed in the micrograph of the upper side of the cyst (Fig. 7). The primordium of the ciliature is also seen (PA). The bundle of fibers and the kinetosomes of the ciliate that are at the lower side of the doublets can not easily be seen (Figs. 3, 6, F', PA'). In these figures and especially in figures 8-10, two main groups of kinetosomes can clearly be distinguished. One of these is the primordium of the adoral zone of the membranelles that consists of many kinetosomes gathered together (Figs. 3, 7, PA, PA', Figs. 9, 10, AZM). They begin to organize themselves into membranelles at the top of this agglomeration (Fig. 9 arrow). The second group appears as several rows of kinetosomes related to the fibers (Figs. 3, 8 RK). As morphogenesis progresses, basal bodies move away from each other and become spaced along the positioning fibers. The streak of kinetosomes splits into small groups which form the fronto-ventral and transverse cirri (Fig. 11, VT). The undulating membrane appears at the base of cirral rows. It forms the buccal cirrus at its anterior end later. When cortical morphogenesis is almost completed the extraordinary points of the macronuclei retract and both macronuclei divide twice. Fig. 11 shows the macronuclei after the first division. Cortical morphogenesis of the doublet is finished. After the second macronuclear division the doublet has eight macronuclei, four in each component (Fig. 12). The two bundles of fibers disappear when cortical morphogenesis is completed.

There are also important changes at ultrastructural level. The macronuclear envelope has chromatin attached to its inner side during the first moments of



Figs. 1-3. Schematic drawings of three steps of the development of both macronuclei during excystment of *Onychodromus acuminatus* doublets. 1 - Cyst in the earliest phase of excystment. The macronuclei are in form of lentils and have condensed nucleoli. 2 - Cyst in the last phase of excystment. Cortical morphogenesis is nearly finished. There are two adoral zones of membranelles. 3 - Cyst in an intermediate phase of excystment. The macronuclei are in their most enlarged appearance. Macronuclear points (P, P'), bundles of fibers (F, F'), primordia of ciliature (PA, PA')

excystment. In front of the chromatin, membranous structures are observed in the cytoplasm (Fig. 13, arrows). At this stage the macronuclear envelope has few pores, that are difficult to observe. The nucleoli at this stage are formed by strongly intermingled granular and fibrillar material showing a compact appearance (Fig. 13, N). The number of pores increases throughout the excystment. At the stage of retraction of the macronuclear points, the macronuclear envelope is abundantly perforated by pores (Fig. 14, arrows).

As the process continues many nucleoli lose their granular component, appearing as ring-shaped nucleoli in sections at the ultrastructural level. Fig. 15 shows this kind of nucleoli inside two macronuclear points (P). Later, there are many clear spaces in the macronuclei similar in size to the nucleoli seen previously. The cytoplasm has small vesicles and is very condensed during early escystment.

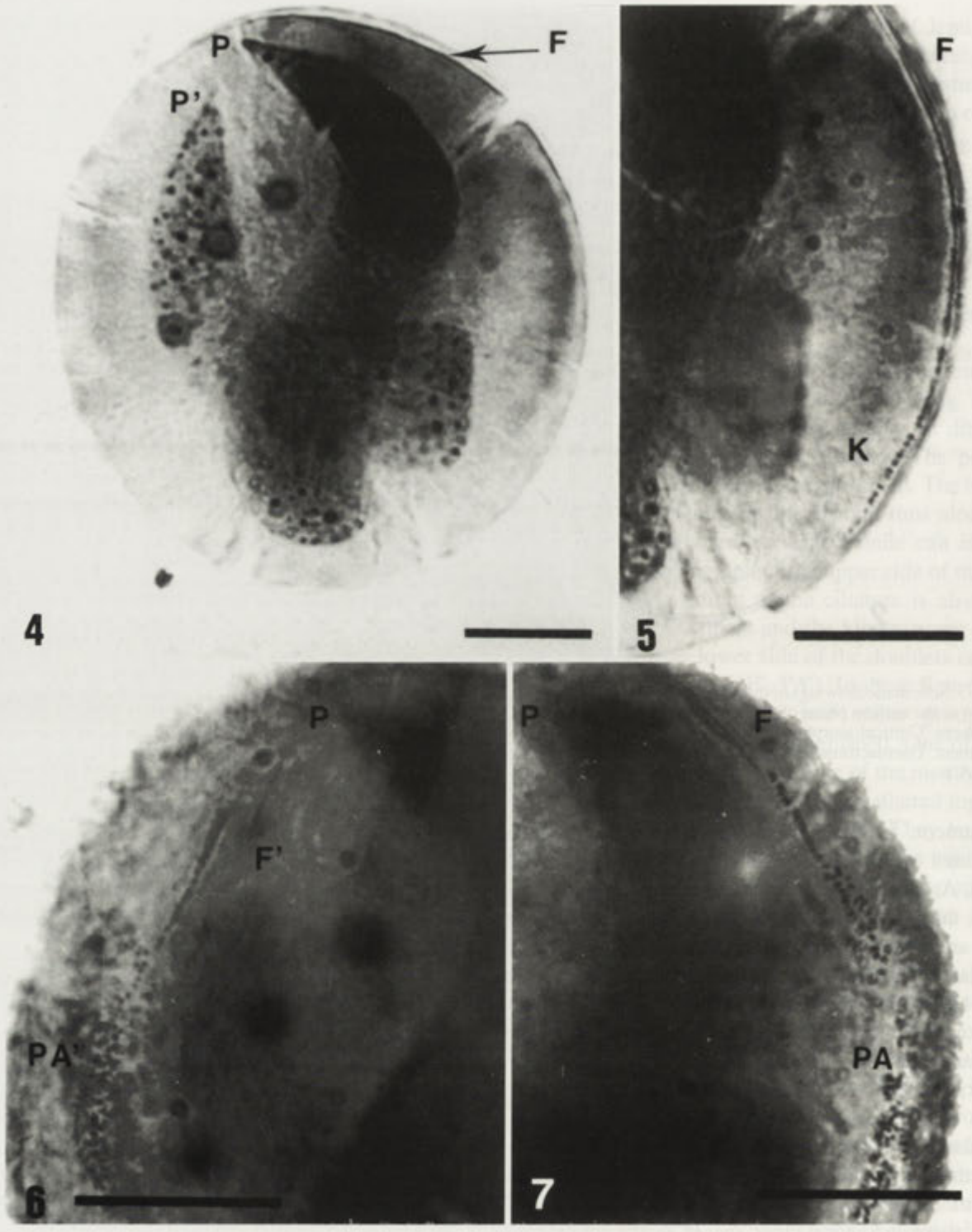
In Fig. 12 the completely developed doublet is represented. This form can revert to the singlet form by longitudinal separation of the two components. The doublets can also divide transversely, giving rise to two doublets. In both cases the doublets may be very symmetrical and have the macronuclei of the two components separate. If, for unknown reasons, the macronuclei fuse simplification of the doublet form occurs.

DISCUSSION

The majority of papers about doublets do not take macronuclear events into account. Some authors noticed that the newly formed doublets had independent macronuclei, and after several divisions the macronuclei became fused. At the same time doublet simplification occurred (Fauré-Fremiet 1948, Nanney et al. 1975, Totwen-Nowakowska 1973).

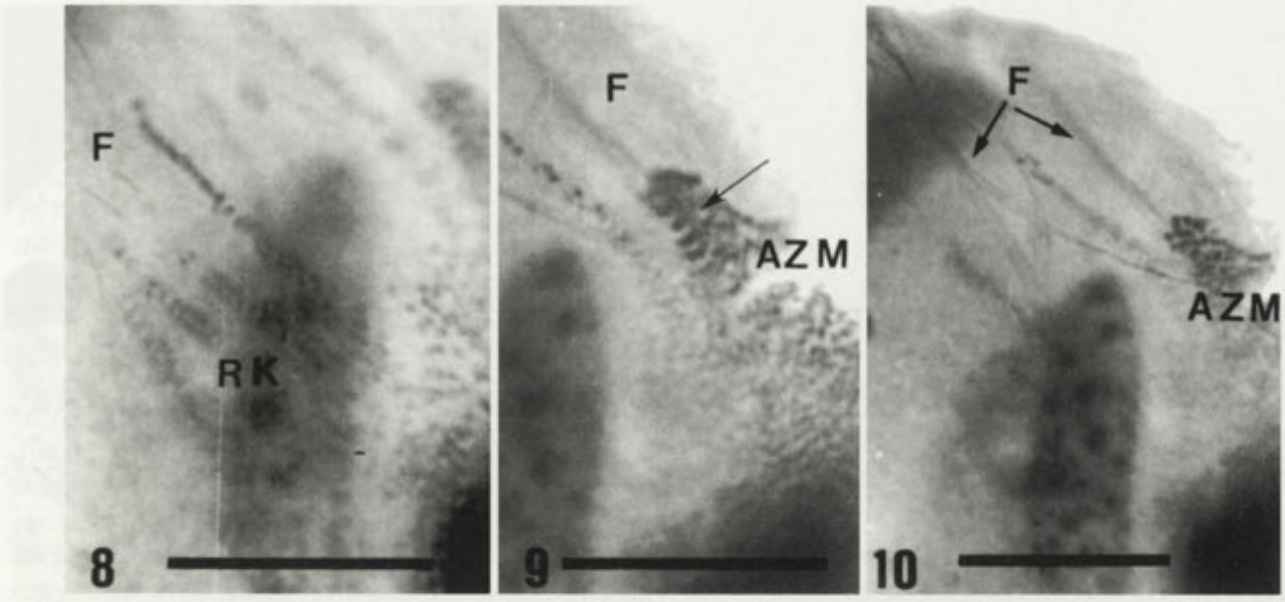
The behavior of the double macronuclear set has not been described before, nor have the two bundles of fibers inside a cyst been shown.

Positioning fibers, which pattern basal bodies, were discovered in *Onychodromus acuminatus* during excystment morphogenesis (Jareño and Tuffrau 1978, 1979). As shown in Results two bundles of fibers are seen



Figs. 4-7. Micrographs from one cyst at four different optical levels. 4 - Micrograph at the central level where the two points (P, P') of the macronuclei can be observed. 5-7 - Micrographs at three different levels where fibers (F, F') kinetosome rows (K) and AZM primordia (PA, PA') are focused. Scale bars : 20 µm

starting close to the two macronuclear points. When a single cell excysts, one bundle of fibers is formed. In some occasions, the bundle has been observed arising from a small body (Jareño 1984). Moreover, some images of excysting ciliates suggest an association between the beginning of the bundle of fibers and the macronuclear point (i.e. Figs 5 and 6, Jareño 1984), but up till now evidence has not been obtained.



Figs. 8-10. Micrographs at three different levels of the same primordium of ciliature at a later stage of excystment. 8 - The rows of kinetosomes (RK) of the future fronto-ventral and transverse cirri can be seen. 9 - The arrow marks the first assembled membranelles. Fibers (F). Adoral zone of membranelles (AZM). 10 - Fibers are clearly observed (F). Scale bars: 20 μm

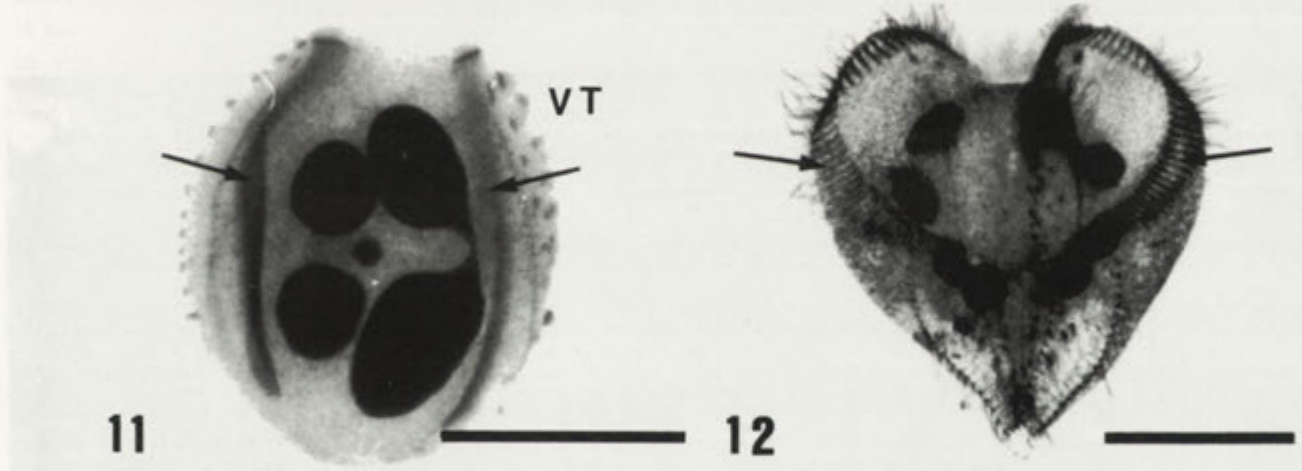


Fig. 11. A doublet that has nearly finished cortical morphogenesis. The two adoral zones of membranelles are marked with arrows. The macronuclei are finishing the first division. Fronto-ventral-transverse cirri (VT). Scale bar: 100 μm

Fig. 12. A completely developed doublet. Arrows mark adoral zones of membranelles. Each component of the doublet after the second division has four macronuclei. Scale bar: 100 μm

Kinetosomes at the light microscope level appear in narrow relation, both spatial and temporal to the fibers which start at the apical pole. Primordia of the fronto-ventral and transverse cirri are formed by rows of kinetosomes that run along the positioning fibers.

Studies at the ultrastructural level, have shown transient microtubules inside the macronuclear points (Jareño 1985) and surrounding the nucleoli (Jareño 1988) during excystment in *Onychodromus acuminatus*. In the cytoplasm, microtubules that could respresent the positioning fibers, have been seen (unpublished results), but more studies are necessary because parallel subpellicular microtubules (typical of the vegetative ciliates) also appear during excystment (Jareño 1980). Transient microtubules, possibly involved in morphogenetic movements, have been described in division morphogenesis (Jerka-Dziadosz 1980) and in a recent abstract (Fleury and Laurent 1991) about zygocyst excystment. Macronuclear changes have not been implicated in these last processes.

Macronuclear events similar to those described here are not known in other ciliates. A similar series of macronuclear events occurs during excystment of single organisms of the same species (Jareño 1984). The main

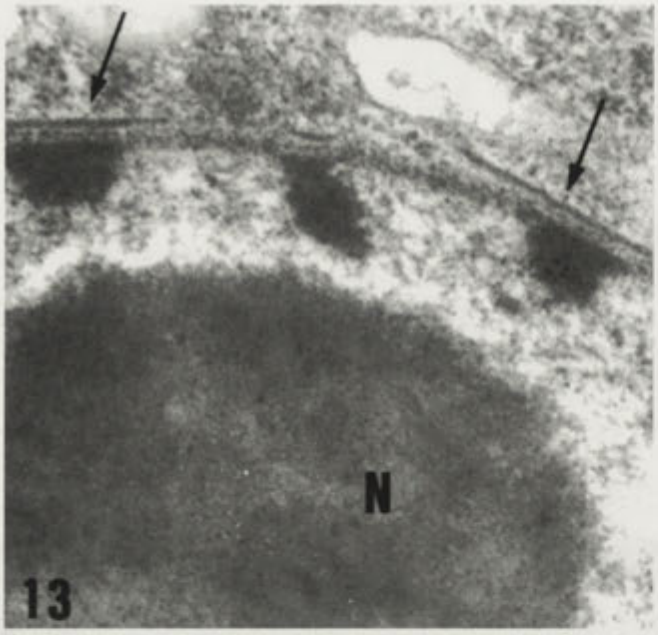


Fig. 13. Electron micrographs of a fragment of a macronucleus during the first moments of excystment. The macronuclear envelope has chromatin attached at the inner side and additional membranes on the outer side (arrows). Section of a condensed nucleolus (N), X 72,000

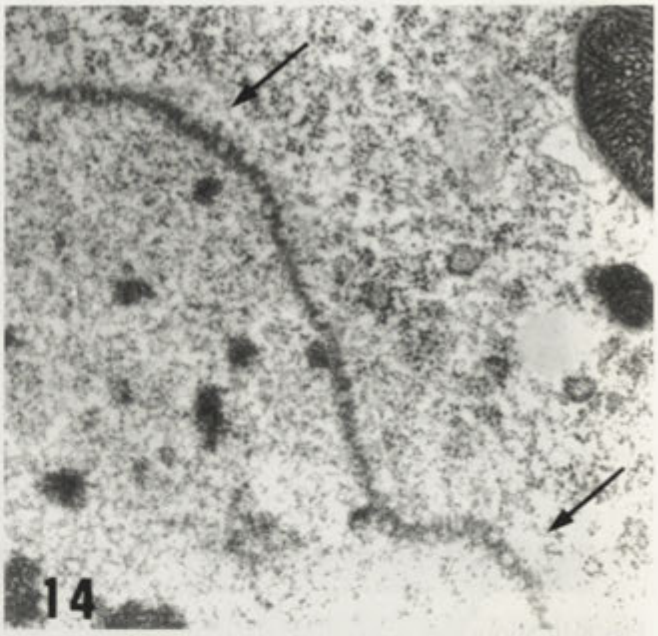


Fig. 14. Fragment of a macronucleus during the last phase of excystment. The macronuclear envelope is abundantly perforated by pores (arrows), X 78,000

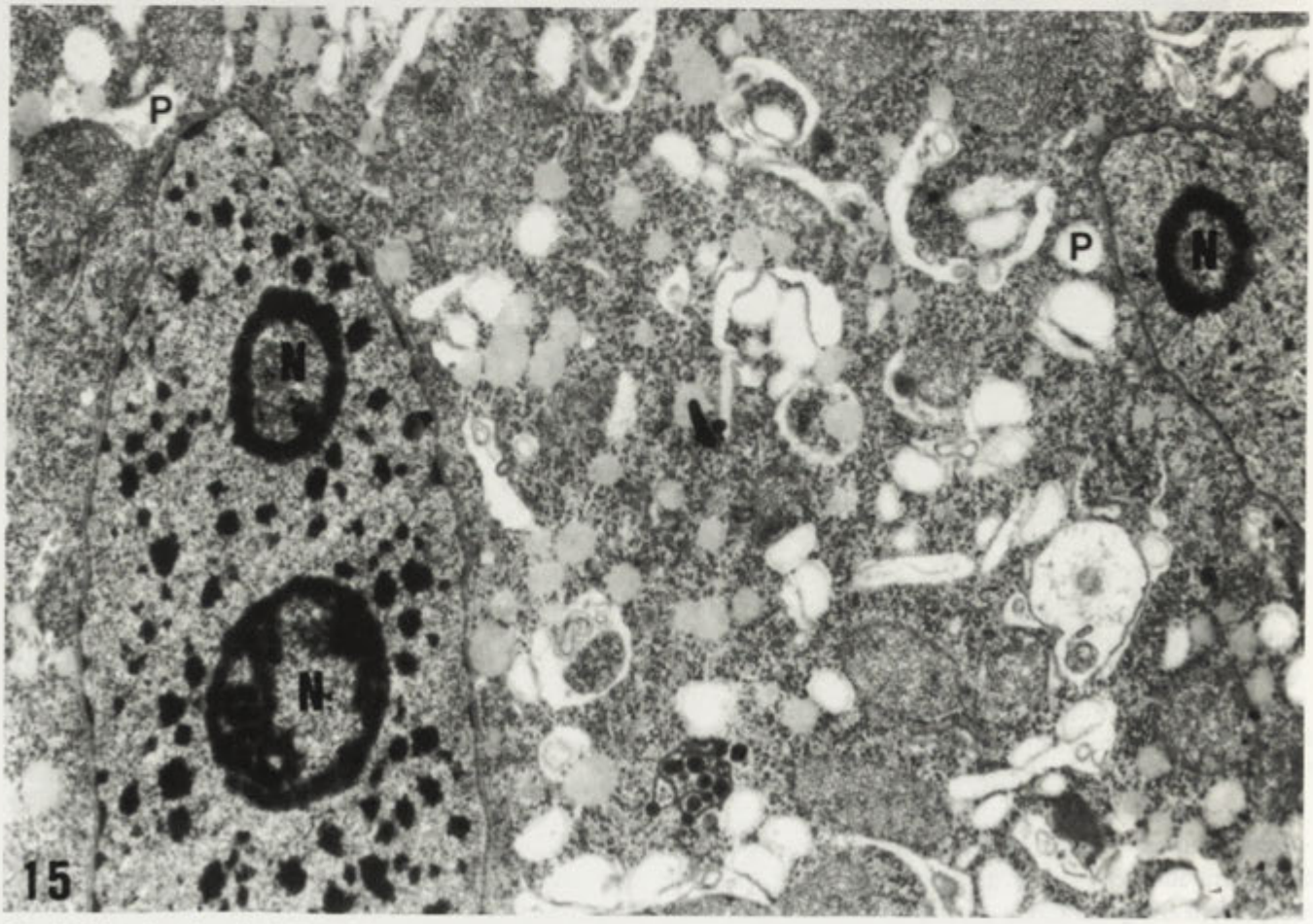


Fig. 15. Ring-shaped nucleoli (N) inside two macronuclear points (P) of a doublet in an intermediate phase of development, X 22,000

difference is the existence of two macronuclei in the developing doublets.

During the excystment, the macronuclei appear to be very active nuclei, not only by their extraordinary lengthening and retraction but also by the changes in their envelope and nucleoli. The number of macronuclear envelope pores increase considerably, favouring nucleocytoplasmic transport.

The ultrastructural changes of the nucleoli are very similar to those occurring in other animal species during processes (i.e. oogenesis) where a high nuclear activity is required (Acevedo and Coimbra 1980, Goessens 1984), and occurs also in single cells of *Onychodromus acuminatus* (Jareño 1988). The disappearance of nucleoli has been described in ciliates during processes such as cell division and conjugation but not during excystment (Raikov 1982).

Of course preexisting ciliatures can play a significant role in primordia formation when they exist (Iftode et al. 1989, Jerka-Dziadosz 1974, Sonneborn 1975) but this is not the case, because there are no cortical structures in the naked cytoplasm of the encysted ciliates of *Onychodromus acuminatus*.

At least during excystment of this species, it is reasonable to think that the macronuclei have the potentiality of initiating and globally patterning morphogenesis. Thus, information for a complete set of ciliature must be transmitted by macronuclear activity in early excystment. In this regard, the experiments by Grimes and Hammersmith (1980) with "humped" cells of *Oxytricha* are very interesting. These cells are incomplete doublets induced experimentally. They are, according to these authors, typical single cells with additional rows of cirri located mid-dorsally. The supernumerary dorsal rows of cirri in these cells, that are transmitted to fission products, are lost when the cells encyst. The excysted ciliates always have the normal single phenotype, indicating that the macronucleus is able to have information for, and the possibility of, inducing a complete set of ciliature; however, it has no information about abnormal cirri remaining from cortical damage. Unfortunately, the authors do not describe the appearance of the macronuclei and their behavior during the experiments.

On the other hand, Totwen-Nowakowska (1973) has shown that removal of macronuclei in one component of *Stylonychia mytilus* doublet evoked always the resorption of this component, although the cortical structures and the AZM remained intact. In this species such as in *Onychodromus acuminatus* the macronuclei seem to have an important role in the maintenance of the doublet condition.

In disagreement with this conclusion, Grimes (1973, 1982) claims that there exists a ventral "determinative region" that would be double in the cysts of doublets of *Oxytricha* and single in cysts of one cell. This region would be able to be inherited and to divide.

These two characteristics are typical of the macronucleus, and we think that the study of the macronuclear events during excystment of *Onychodromus acuminatus* can explain and also help to understand many if not all phenomena related to doublets.

This paper stresses the importance of the macronuclei during the morphogenesis of excystment in doublets of *O. acuminatus* presenting for the first time morphological and ultrastructural data in ciliates. This report does not pretend, of course, to resolve the important biological problems that the development of doublets raises, but only to show the existence of macronuclear events both spatial and temporarily related to superficial fibres and cortical morphogenesis during early excystment of a doublet.

More insight into this species, and studies in other ciliates that have similar process of excystment are necessary to confirm this interpretation.

REFERENCES

Acevedo C., Coimbra A. (1980) Evolution of the nucleoli in the course of oogenesis in viviparus teleost (Xiphophorus helleri). Biol. Cell. 38: 43-48.

Calkins G. N. (1925) *Uroleptus mobilis*. The history of double organisms. J. Exp. Zool. 41: 191-213.

Fleury A., Laurent M. (1991) Abst. Int. Conf. Cell. Mol. Biol. Ciliates. 21, Toledo (Spain).

Fauré-Fremiet E. (1948) Doublets homopolaires et régulation morphogénétique chez le cilié *Leucophrys patula*. Arch. Anat. Microsc. Morphol. Exp. 37: 183-203.

Frankel J. (1962) The effects of heat, cold and p-fluoro-phenylalanine on morphogenesis in synchronized *Tetrahymena pyriformis GL*. CR Trav. Lab. Calsberg 33: 1-52.

Goessens G. (1984) Nucleolar structure. Int. Rev. Cytol. 87: 107-158.

Grimes G. W. (1973) An analysis of determinative difference between singlets and doublets of *Oxytricha fallax*. Genet. Res. 21: 57-66.

Grimes G. W. (1982) Pattern determination in hypotrich ciliates. Amer. Zool. 22: 35-46.

Grimes G. W., Hammersmith R. L (1980) Analysis of the effects of encystment and excystment on incomplete doublets *Oxyticha fallax*. J. Embryol. exp. Morph. 59: 19-26.

Iftode F, Cohen J., Ruiz F., Torres A., Chen-Sun L., Adoutte A., Beison J. (1989) Devslopment of surface pattern during division in *Paramecium*. I. Mapping of duplication and reorganization of cortical cytoskeletal structures in the wild type. Development 105: 191-211.

Jareño M. A. (1977) Enkystement conjoint chez un cilié hypotriche. Protistologica 13: 187-194.

Jareño M. A. (1980) Observations sur l'ultrastructure du dékystement chez *Onychodromus acuminatus*. J. Protozool. 27, 83A.

Jareño M. A. (1984) Macronuclear events and some morphogenetic details during the excystment of *Onychodromus acuminatus* (Ciliophora, Hypotrichia). J. Protozool. 31: 489-492.

Jareño M. A. (1985) Etude ultrastructurale de l'enkystement et du dékystement chez *Onychodromus acuminatus* (Ciliata, Hypotrichida). Protistologica 21: 313-321.

Jareño M. A. (1988) Nucleolar structural changes during the excystment of *Onychodromus acuminatus* (Ciliophora, Hypotrichia). Arch. Protistenkd. 138: 191-198.

Jareño M. A., Tuffrau M. (1978) Positionnement et morphogenèse sous kyste chez un cilié hypotriche du genre *Onychodromus*. J. Protozool. 25, 55A.

Jareño M. A., Tuffrau M. (1979) Le positionnement des primordiums cinétosomiens et la morphoganèse sous kysts chez le cilié hypotriche *Onychodromus acuminatus*. Protistologica 15: 597-605.

Jerka-Dziadosz M. (1974) Cortical development in *Urostyla*. II. The role of poaitional information and preformed structures in formation of cortical pattern. Acta Protozool. 12: 239-274.

Jerka-Dziadosz M. (1980) Ultrastructural study on development of the hypotrich ciliate *Paraurostyla weissei*. I. Formation and morphogenetic movements of ventral ciliary primordia. Protistologica 16: 571-589.

Jerka-Dziadosz M. (1983) Ths origin of mirror-image symmetry doublet cells in the hypotrich ciliate *Paraurostyla weissei*. Roux's Arch. Dev. Biol. 182: 179-188.

Nanney D. L., Chow M., Wozencraft B. (1975) Considerations of the symmetry in cortical integration of *Tetrahymena* doublets. J. Exp. Zool. 193: 1-14.

Raikov I. B. (1982) The protozoan nucleus. Cell. Biol. Monogr. 9: 1-467, Springer Verlag, Wien.

Shi X., Frankel J (1990) Morphology and development of mirror-image doublets of *Stylonychia mytilus*. J. Protozool. 37: 1-3.

Sonneborn T. M. (1975) Positional information and nearest neighbor interactions in relation to spatial patterns in ciliates. Ann. Biol. 14: 565-584.

Totwen-Nowakowska I. (1965) Doublets of *Stylonychia mytilus* (O.F.M.) evoked by action of thermic shocks. Acta Protozool. 3: 356-361.

Totwen-Nowakowska I. (1973) Conditions of stability of double system in the post-traumatic regeneration of doublets of *Stylonychia mytilus*. Acta Protozool. 12: 53-70.

Tuffrau M. (1976) Perfectionnements et practique de la technique d'imprégnation au protargol des infusoires ciliés. Protistologica 3: 91-98.

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Identification of Ciliary Membrane Proteins Closely Related with the Mating Reactivity of *Paramecium caudatum*

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Summary. Cilia of *Paramecium caudatum* were isolated by the $MnCl_2$ method from mating-reactive mature cells of the early stationary phase of growth, mating-nonreactive immature cells of the stationary phase of growth, mating-nonreactive proliferating mature cells, and mating-nonreactive mature cells of the late stationary phase of growth. Their ciliary membranes were then prepared by the urea-EDTA method and these polypeptide compositions were compared by two-dimensional -SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE) and silver staining. We found four polypeptides (pI 5.5-6.0, MW 70-92 kD) which existed only in mature cells of two complementary mating types regardless of the presence of the mating reactivity. However, one of the polypeptides (pI 5.5, MW 92 kD) was missing in mating-reactive mature cells in both mating types. Since these four polypeptides were present in trace amounts in the 2D-SDS-PAGE gel of axonemal proteins, it was suggested that these polypeptides were ciliary membrane proteins. The results indicate that some of them may be involved in the mating-type substances of both mating types, and that the disappearance of a specific polypeptide from the mating-type substances may create the active site of the mating-type substances.

Key words. Ciliary membrane proteins, *Paramecium caudatum*, mating reactivity.

INTRODUCTION

Sexual cell recognition between cells of complementary mating types of *Paramecium* is mediated by specific macromolecules of the ventral surface cilia (Hiwatashi 1961, Cohen et al. 1963, Byrne 1973). These molecules are known as mating-type substances, and are thought to be proteins which are tightly bound to ciliary membranes (Kitamura and Hiwatashi 1976, 1978, 1980; Takahashi et al. 1974, Watanabe 1977). When cells of complementary mating types are mixed under appropriate conditions, they soon stick together by their ventral surface cilia and form a large cell aggregate. This agglutinative adhesion is called the mating reaction (Sonneborn 1939). Although many investigators have tried to detect and isolate the mating-type substances in a molecular form, no one has yet succeeded, because the mating-type substance activity (sexual cell-to-cell agglutination inducing activity) could not be maintained when the ciliary membranes were solubilized. The simplest systems exhibiting mating-type substance activity so far obtained are those of two kinds of ciliary membrane vesicles in *P. caudatum*; one has a diameter of 100-150 nm and is obtained by treatment of mating-reactive cilia with urea and EDTA, and the other has a diameter of 50-100 nm and is reconstituted from the

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lithium diiodosalicylate (LIS)-soluble fraction of the urea-EDTA vesicles by dialysis (Kitamura and Hiwatashi 1976, 1980). However, an attempt to find differences in polypeptide compositions between urea-EDTA membrane vesicles of complementary mating types, using one-dimensional -SDS- polyacrylamide gel electrophoresis (SDS-PAGE), failed (Kitamura and Hiwatashi 1976). On the other hand, using two-dimensional -SDS- polyacrylamide gel electrophoresis (2D -SDS-PAGE) of ciliary membranes prepared without the use of detergents, Adoutte et al. (Adoutte et al. 1980) found a protein of molecular weight 31 kD which varied in its amount in good correlation with cellular mating reactivity in *P. tetraurelia*. They suggested the possibility that this protein might be in some way involved in the mating reaction. However, whether the variation in the amount of the 31 kD protein was really correlated with the mating reactivity of the ciliary membranes and whether the 31 kD protein was mating type specific or a common to both mating types has not yet been examined. Therefore, the possibility of the 31 kD protein participating in mating-type substances is still unclear.

In the present study, we prepared four kinds of ciliary membranes from cells of two complementary mating types of different stages in the life cycle and the culture of *P. caudatum*; mating-nonreactive immature cells of stationary phase of growth, mating-nonreactive proliferating mature cells, mating-reactive mature cells of early stationary phase of growth and mating-nonreactive mature cells of late stationary phase of growth. After confirming that the ciliary membranes derived from the mating-reactive cilia could induce agglutination among living cells of the complementary mating type, their polypeptide compositions were compared with those of other ciliary membranes using 2D-SDS-PAGE.

MATERIALS AND METHODS

Culture Conditions

Paramecium caudatum syngen 12, strains 19a and 18b were used. In this syngen, polymorphisms were found in both mating types, E and O. E^{12a} is complementary both to type O^{12a} and O^{12b}, but E^{12b} is reactive only with type O^{12a} (Tsukii 1988). Mating types of 19a and 18b are O^{12a} and E^{12b} respectively. These strains were F_1 progenies of a cross between two natural stocks, Ohuchi (mating type O^{12a}) and Ishin (mating type E^{12b}), collected in Yamaguchi, Japan. To minimize the differences in genetic backgrounds between complementary mating type cells, we used these sibling strains for experimental animals. The culture medium was 1.25% (w/v) fresh lettuce juice in Dryl's solution, bacterized with *Klebsiella pneumoniae* a day before use (Hiwatashi 1968). The cultures were maintained at 25°C.

Maintenance of Cell Lines

Two exconjugant cell lines (strains 19a and 18b) were maintained as follows. About 20 fissions after conjugation, single cells derived from different exconjugant lines were isolated to test tubes containing 2 ml fresh culture medium. On successive days, culture medium was added in 4, 10 and 10 ml aliquots. When these lines reached the stationary phase one day after final feeding, several hundred cells were inoculated into 2 ml culture medium in new test tubes and then 4 ml, 10 ml and 10 ml of fresh medium were added on successive days. Cells underwent about 15 fissions in the first test tube, but about 4 fissions at and after the second tube. Therefore, the fission age of the clone was calculated as the sum of the number of transplantations x 4 plus 15. Strains 19a and 18b exhibited mating reactivity after the 13th transplantation (about 67 fissions after conjugation) and their mating types were found to be O^{12a} and E^{12b} respectively. One day after the final feeding (early stationary phase of growth), mating reactivity increased to the maximum intensity, but the reactivity decreased at 2 days after the final feeding and completely disappeared at 4 days after the final feeding (late stationary phase of growth). Immature and mature cells of strains 19a and 18b used were about 39-51 fissions and 71-91 fissions in their clonal age. Mating-nonreactive immature cells were harvested from the early stationary phase of growth in the immaturity period. Mating-reactive and non-reactive mature cells were harvested from the early and the late stationary phases of growth, respectively. Mating-nonreactive proliferating mature cells were obtained as follows. A 10 l of culture in the early stationary phase of growth was mixed with an equal volume of fresh culture medium in a 20 l polycarbonate tank and cultivated for 10 hr at 25°C. Using this method, the division index increased from 0% to about 15% (Fujishima and Hori 1989).

Isolation of Cilia

Cilia were isolated by the $MnCl_2$ method (Fukushi and Hiwatashi 1970) with slight modifications: about a 20 l of culture was filtered through 8 layers of gauze to remove gross debris and the cells were harvested by continuous centrifugation at 700g for 10 min at 2°C, washed 3 times with 10 mM Tris-HCl, pH 6.8 (instead of pH 7.6 of original method) by centrifugation at 300g for 1 min, transferred into 50 ml of 10 mM Tris-HCl, pH 6.8 containing 70 mM $MnCl_2$ and chilled with stirring at 4°C. After 30 min, cell bodies were sedimented by centrifugation at 300g for 1 min and the supernatant containing detached cilia was filtered through filter paper (Advantec Toyo, No. 2) at 4°C to remove remaining cell bodies and trichocysts. The cilia were recovered by centrifugation at 12,000g for 10 min at 2°C, washed twice by the same centrifugation with 10 mM Tris-HCl, pH 6.8, and frozen at – 85°C until use.

Before freezing of the cilia, an aliquot of the mating-reactive cilia was added to the external medium of mating-reactive cells of the complementary mating type and it was confirmed that the detached cilia could induce sexual cell agglutinations in the tester cells.

Isolation of Ciliary Membrane Vesicles

Ciliary membrane vesicles were prepared by the urea-EDTA method (Kitamura and Hiwatashi 1976). Isolated cilia were suspended in 2 ml of medium containing 2 M urea, 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.3 for 60 min at 4°C and centrifuged at 9,000 g

for 10 min at 4°C. The pellet (crude axonemal fraction) was used for purification of axonemes as described later, and the supernatant was then filtered through a 0.22 µm membrane filter (Advantec Toyo) and dialysed against 1 l of 10 mM Tris-HCl, pH 7.3 using dialysis tubing (Union Carbide Co.) for 24 hr, changing the buffer every 8 hr. The dialysate was then centrifuged at 105,000 g for 60 min at 4°C and the pellet (urea-EDTA ciliary membrane fraction) was frozen at – 85°C until use. Mating-reactivity of the ciliary membranes was tested as previously described.

Isolation of Axonemes

Cilia were obtained from mating-reactive mature cells of strain 18b in the stationary phase of growth and the crude axoneme fraction were obtained by Urea-EDTA from the detached cilia, as previously described. The crude axoneme fraction was then further purified using the Watanabe's procedure (1977) as follows. The crude axonemes were extracted 3 times for 15 min each with 5 ml of Triton-EDTA (1% [v/v] Triton X100, 1mM EDTA, 10 mM Tris-HCl, pH 7.4) in an ice bath. After each extraction, the cilia were collected by centrifugation at 9,000 g for 10 min at 4°C. During the extraction, the preparation was sometimes drawn in and out of a Pasteur pipette. The extracted ciliary pellet was then washed with the same centrifugation with 10 mM Tris-HCl, pH 7.4, and then the pellet was suspended in 2 ml of 10 mM Tris buffer, layered over 2.5 ml of 40% (w/v) sucrose in 10 mM Tris buffer and centrifuged at 15,000 g for 30 min at 4°C. The resulting pellet was washed once by the same centrifugation with 10 mM Tris buffer, and stored at – 85°C until use.

Two - Dimensional - SDS - Polyacrylamide Gel Electrophoresis (2D-SDS-PAGE)

Isolated urea-EDTA ciliary membranes and axonemes were lysed in the solubilizing solution containing 8.5 M urea, 2% (v/v) Nonidet P-40, 2% (w/v) ampholine and 5% (v/v) 2-mercaptoethanol. Insoluble materials were removed by centrifuging at 5,000 g for 5 min. 2D-SDS-PAGE of polypeptides was carried out essentially as described by O'Farrell (1975). The first dimension was cast as a 10 cm column in 2.5 mm diameter glass tubes using 1.6% (w/v) of pH 5-8 and 0.4% (w/v) of pH 3.5-10 ampholine (Pharmacia LKB). After pre-running at 200, 300 and 400 V for 15, 30 and 30 min, respectively, each tube was loaded with 21 µg of urea-EDTA ciliary membrane protein and 50 µg of axonemal protein, and run at 400 and 800 V for 16 and 1 hr, respectively. The isoelectric focusing gels were equilibrated with Laemmli's buffer (Laemmli 1970) containing bromophenol blue. In the second dimension, polypeptides were electrophoresed according to molecular weight on a SDS-polyacrylamide slab gel consisting of a stacking gel (120 x 20 x 1 mm) containing 3% (w/v) SDS and a separation gel (120 x 100 x 1 mm) containing 10% (w/v) SDS at 15 mA and 30 mA constant current, respectively, until the tracking dye moved to 5 mm from the bottom of the gel. Polypeptides in the gel were stained with silver using a silver stain kit (Wako). A spot of less than 0.2 µg protein can be detected by this staining.

Protein content of the sample to be loaded to 2D-SDS-PAGE was measured spectrophotometrically with a Protein Assay (Bio-Rad). Molecular weights were measured using a Pharmacia low molecular weights kit (Pharmacia LKB); phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and α-lactalbumin (14 kD). Molecular weights of sample proteins were extrapolated from a plot of the Rf vs. log molecular weight of the standards (Weber and Osborn 1969). To determine the pH gradient of the gels, an isoelectric point (pI) markers kit (pI range, 4.7-10.6) (Pharmacia LKB) was used.

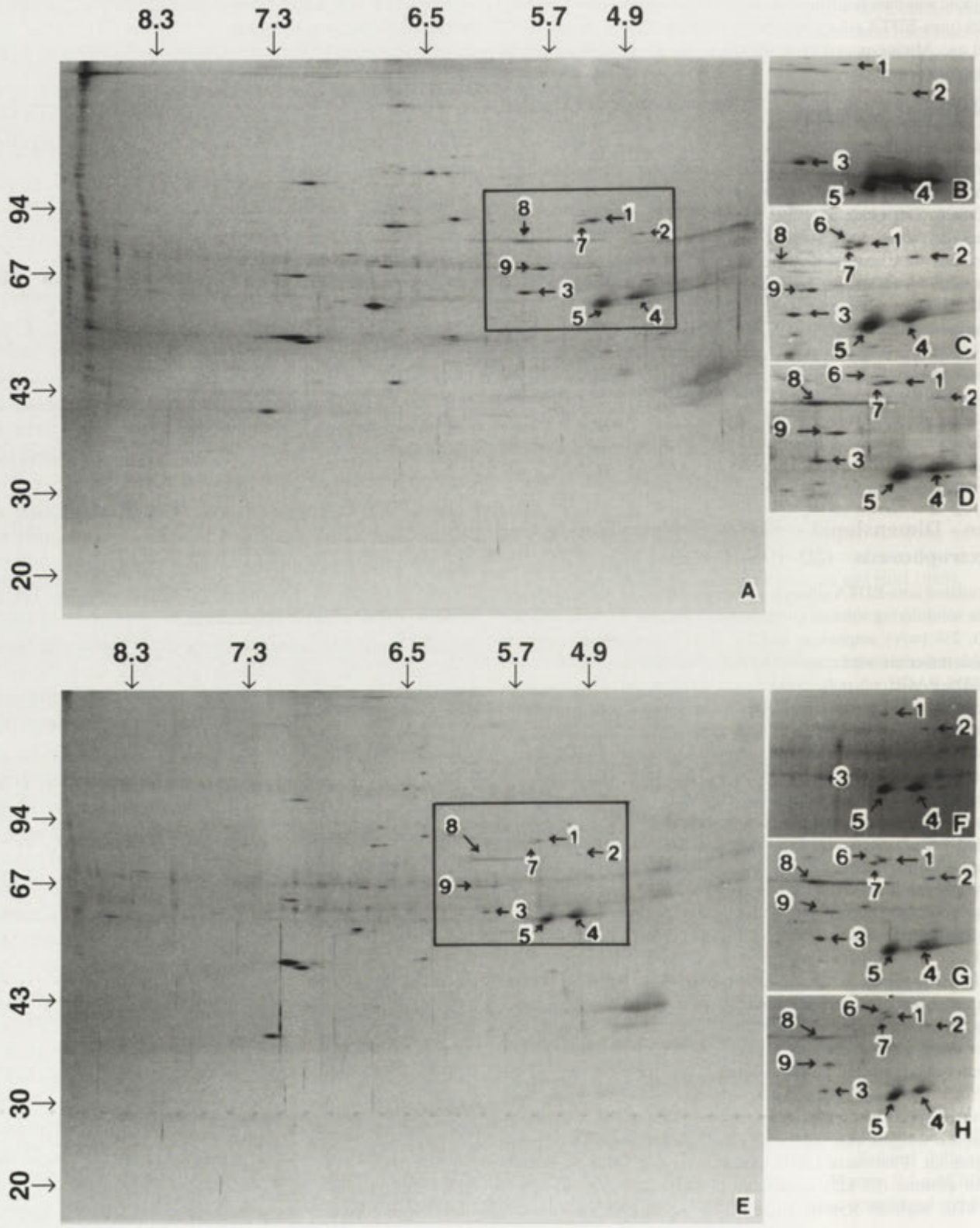
2D-SDS-PAGE was repeated at least 3 times in each sample and its reproducibility was confirmed.

RESULTS

Changes of ciliary membrane polypeptides in clonal age and culture age

The polypeptide compositions of ciliary membranes prepared by the urea-EDTA method were compared by 2D-SDS-PAGE among mating-nonreactive immature cells of the stationary phase of growth, mating-nonreactive proliferating mature cells, mating-reactive mature cells of the early stationary phase of growth and mating-nonreactive mature cells of the late stationary phase of growth. Mating reactivity of the detached cilia and ciliary membranes obtained from the mating-reactive mature cells of the early stationary phase of growth were confirmed by mixing them with mating-reactive living cells of the opposite mating types (tester cells) and inducing cells into mating clumps. The detached cilia and the ciliary membranes could retain the mating-type reactivity even if these were kept at – 85°C for one month. Figs. 1 and 2 show these four samples of mating type E^{12b} and O^{12a}, respectively. High molecular weight regions of the gels, which included immobilization antigens, were cut off from Fig. 1. In all samples, about 80-90 polypeptides were resolved on gels of the ciliary membrane fraction. In an acidic area of pI 4.8-6.2 and molecular weights 54-94 kD, we found four polypeptides that appeared depending on clonal age and culture age of the cells. In the squared area of Figs. 1 and 2, the major polypeptides including the four polypeptides were numbered. Nos. 1-5 were commonly present in both mating types notwithstanding the differences of mating-type, clonal age and culture age. Nos. 4 and 5 were tubulin subunits β and α of MW 54 kD, pI 5.0-5.1 and MW 52 kD, pI 5.3-5.4, respectively, because the two polypeptides were the most predominant polypeptides in a 2D-SDS-PAGE gel of axonemal proteins of strain 18b (Fig. 2). However, four polypeptides (Nos. 6-9) in Fig. 1 appeared only in ciliary membranes of mature cells and were lacking in those of immature cells in both mating types. Molecular weight (MW) and pI of Nos. 6-9 polypeptides were as follows; MW 92 kD, pI 5.5 and MW 88 kD, pI 5.5 and MW 81 kD, pI 6.1 and MW

70 kD, pI 5.9. It should be noted that No. 6 polypeptide was almost lacking in mating-reactive mature cells of both mating types (Figs. 1A and 1E) although the polypeptide was present in two kinds of mating-nonreactive ciliary membranes of mature cells of both mating types (Figs. 1C, 1D, 1G and 1H). These results suggest that some of these four mature cell-specific polypeptides may be in some way involved in the mating-type



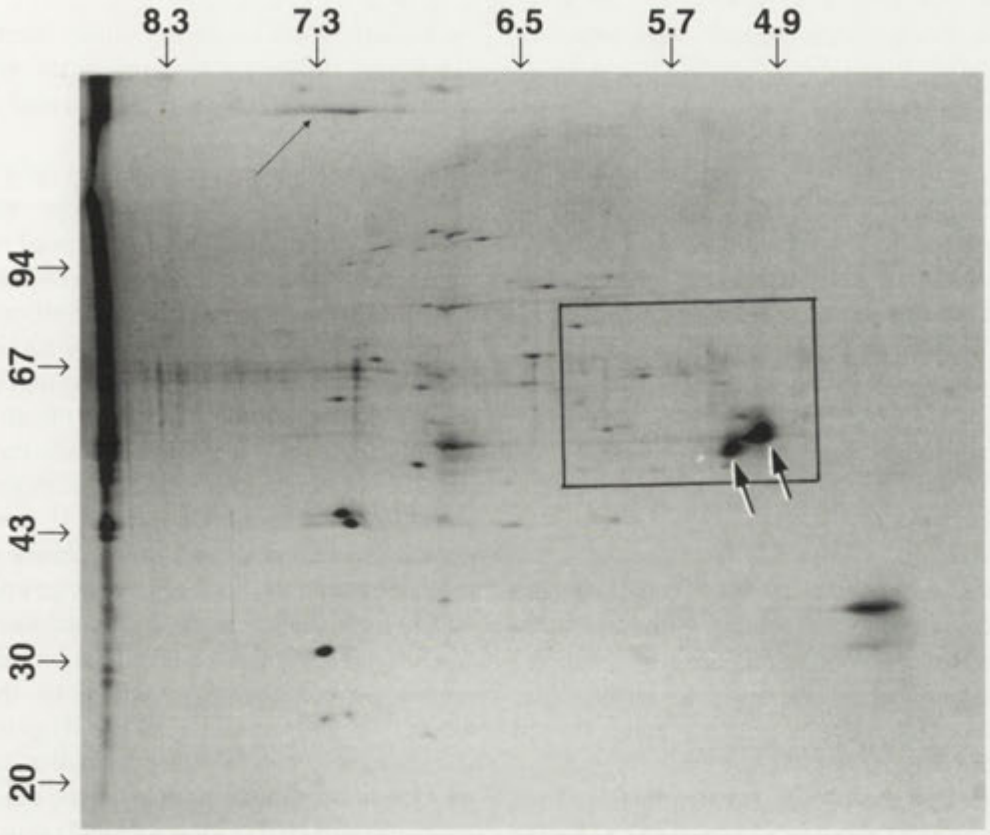


Fig. 2. Two-dimensional-SDS-PAGE gel of axonemes of mating type E^{12b}. Axonemes were isolated from mating-reactive mature cells from the early stationary phase of growth. Gel was run and stained with a silver as described in Materials and Methods section. A squared area corresponds to those in Figs. 1A-1H. Note the large amounts of tubulin subunits (arrows) in the area. An arrowhead shows immobilization antigens derived from contaminated ciliary membranes

substance, and that disappearance of the No. 6 polypeptide from the mating-type substances may play a role in forming the active site of the mating-type substances in the early stationary phase of growth.

In the gel of the axonemal fraction, about 60 polypeptides were recognized, and in which polypeptides Nos. 1-3 and 6-9 were missing or present only in trace amounts, indicating that these polypeptides were involved in ciliary membranes. It seemed that No. 5 (tubulin α) consisted of at least two isotypes (Figs. 1 and 2). The presence of tubulins in the ciliary membrane indicates that a small amount of axonemal components were involved in the ciliary membrane fraction. Similar amounts of contamination of tubulins in the ciliary membrane fraction were also reported in *P. tetraurelia* and *Tetrahymena pyriformis* (Adoutte et al. 1980). Similarly, contamination of a small amount of immobilization antigens was also detected in the axonemal fraction (Fig. 2).

Comparison of polypeptide compositions between ciliary membrane vesicles of two complementary mating types

From cross experiments in *P. caudatum* (Tsukii and Hiwatashi 1983), the reactive site of the mating type E substance is expected to consist of a single polypeptide and that the O substance consists of at least two polypeptides. These polypeptides are expected to be present in ciliary membranes of mating - reactive mature

Fig. 1. Two-dimensional-SDS-PAGE gels of ciliary membranes of mating - type E^{12b} (A-D), and mating - type O^{12a} (E-H). Ciliary membranes isolated from mating-reactive cells of the early stationary phase of growth (A and E), mating-nonreactive immature cells of the stationary phase of growth (B and F), mating-nonreactive proliferating mature cells (C and G), and mating-nonreactive mature cells of the late stationary phase of growth (D and H). (B)-(D) and (F)-(H) correspond to squared areas in (A) and (E). Major polypeptides in the area were numbered. Note that the four polypeptides (Nos. 6-9) appeared only in the ciliary membranes of mature cells. Furthermore, No. 6 polypeptide was lacking in the mating-reactive mature cells of both mating types (Figs. A and E) though the polypeptide was present in the other two membrane vesicles of mature cells of both mating types (Figs. C, D, G and H)

cells (Watanabe 1977). In the present study, however, we could not find such matingtype specific polypeptides at least among the major spots on our 2D-SDS-PAGE gels. This suggests that the polypeptides amounts might be very low in the gels, or these polypeptides might exist in the high molecular weight region larger than 200 kD or in the low molecular weight region less than 10 kD on our gels, or pIs of these polypeptides might be less than 4 or more than 8, because polypeptides in these regions could not be well visualized by O'Farell's 2D-SDS-PAGE using 10% uniform SDS-PAGE slab gels.

DISCUSSION

Generally, mating-type substances of *P. caudatum* are believed to be protein which appears in the ciliary membranes when proliferating cells are starved in the early stationary phase of growth of the maturity period (Hiwatashi 1988). However, the present study showed another possibility on an expression mode of mating-type substance reactivity. That is, some subunits which consist of mating-type substances may appear in the ciliary membranes when cells entered the maturity period notwithstanding with or without the presence of the mating-type reactivity, and disappearance of one of them may play a role in forming an active site for the mating-type substances in the early stationary phase of growth. We found 4 mature cell-specific ciliary membrane polypeptides using 2D-SDS-PAGE in both of the complementary mating types. One of them disappeared from the ciliary membranes when the cells expressed the mating-type reactivity. In *P. caudatum*, this is the first report to show that polypeptide compositions of ciliary membranes differ in clonal age and culture age. However, when considering the possibility of a completely different function, the four maturity-period specific polypeptides may be related to the modifications occurring in cell physiology during the immaturity-maturity transition period. To confirm whether these polypeptides really are related to the mating-type substance, we are now attempting to obtain monoclonal antibodies specific for these polypeptides. If some of the proteins are involved in the mating-type substances, it is expected that antigens are detected only in the ventral surface cilia by indirect immunofluorescence microscopy and the antibodies can block the mating-type reactivity of the living cells. Furthermore, to determine whether Nos. 6-9 polypeptides are exposed on the outer surface of the ciliary membrane, we are attempting to examine sensitivities of the proteins against proteases added to the external medium of the cells.

Experiments were done on cells grown in bacterized lettuce medium. Light microscope observations of washed cells and isolated cilia showed only very small numbers of bacteria even if the cilia were prepared from cells in the log phase of growth. Furthermore, since the density of bacteria, axonemes, and trichocysts is much higher than that of the ciliary membranes, it is expected that they separate completely from the membranes under strong centrifugal force. Therefore, contamination of bacterial proteins during ciliary membrane preparation seems to be a negligible amount if present. The only common contaminant in the membrane preparations is a small amount of membrane vesicles enclosing a small part of an axoneme as shown by Adoutte et al. (1980). Occurrence of this contamination in our ciliary membrane preparation is shown by the presence of tubulin subunits in 2D-SDS-PAGE gels of the ciliary membranes. On the contrary, a small amount of proteins which had been major proteins of the ciliary membranes were also detected in the axonemal preparation, indicating a small amount of contamination of the ciliary membranes in the axonemal preparation. This ciliary membrane contamination in axonemal preparation had been shown by transmission electron microscopy (Watanabe 1977).

In this study, we could not find mating-type specific polypeptides. However, we cannot exclude the possibilities that mating-type specific proteins or other maturity-period specific polypeptides might exist among minor polypeptides or in the high molecular weight region of more than 200 kD or in the low molecular weight region of less than 10 kD, or in the acidic region of less than pI 4, or in the basic region of more than pI 8 in our 2D-SDS-PAGE gels. Further analysis of ciliary membrane proteins using gradient slab gels which can cover a wide pH region and wide molecular weight region is now in progress.

Adoutte et al. (1980) reported that a protein of 31 kD, pI 6.8, which was one of the major proteins of the ciliary membranes, was virtually absent in ciliary membranes prepared from cells in exponential growth, but became prominent in the early stationary phase, and then decreased in starved, nonreactive cells which was in good correlation with the cellular mating reactivity of *P. tetraurelia*. In the present study, however, we could not find such a protein in our gels.

In syngen 12 of *P. caudatum*, polymorphisms are known in both mating types, E and O (see Materials and Methods section) (Tsukii 1988). Furthermore, inter-syngen mating reactions occur between cells of mating type O^{12a} of syngen 12 and mating type E^{3} of syngen 3, and between cells of mating type E^{12a} of syngen 12 and mating type O^{13} of syngen 13, suggesting that mating substances are closely correlated among these 3 syngens. Therefore, if polypeptides involved in the mating-type substances were found in syngen 12, which will be useful probes not only for the understanding of subunit structures of complementary mating-type substances, but also for understanding of differentiation of mating type polymorphisms, and evolution of syngens at the molecular level.

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REFERENCES

Adoutte A., Ramanathan R., Lewis R. M., Dute R. R., Ling K.-Y., Kung C., Nelson D. L. (1980) Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. J. Cell Biol. 84: 717-738.

Byrne B. C. (1973) Mutational analysis of mating type inheritance in syngen 4 of *Paramecium aurelia*. Genetics 74: 63-80.

Cohen L. W., Siegel, R. W. (1963) The mating type substances of *Paramecium bursaria*, Genet. Res. 4: 143-150.

Fujishima M., Hori M. (1989) Induction of reinitiation of meiosis in amphibian *Bufo* oocytes by injection of ciliate *Paramecium* extracts. Dev. Biol. 136: 455-458.

Fukushi T., Hiwatashi K. (1970) Preparation of mating reactive cilia from *Paramecium caudatum* by $MnCl_2$. J. Protozool. 17 (Suppl.): 21.

Hiwatashi K. (1961) Locality of mating reactivity on the surface of *Paramecium caudatum*. Sci. Rep. Tohoku Univ. Ser. 4 27: 93-99.

Hiwatashi K. (1968) Determination and inheritance of mating type in *Paramecium caudatum*. Genetics 58: 373-386.

Hiwatashi K. (1988) Sexual recognition in *Paramecium*. In: Eukaryotic cell recognition (Eds. G. P. Chapman, C. C. Ainsworth, C. J. Chatham), Cambridge University Press, Cambridge, pp. 77-91.

Kitamura A., Hiwatashi K. (1976) Mating-reactive membrane vesicles from cilia of *Paramecium caudatum*. J. Cell Biol. 69: 736-740.

Kitamura A., Hiwatashi K. (1978) Are sugar residues involved in the specific cell recognition of mating in *Paramecium*. J. Exp. Zool. 203: 99-108.

Kitamura A., Hiwatashi K. (1980) Reconstitution of mating active membrane vesicles in *Paramecium*. Exp. Cell Res. 125: 486-489.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

O'Farell P. H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem, 250: 4007- 4021.

Takahashi M., Takeuchi N., Hiwatashi K. (1974) Mating agglutination of cilia detached from complementary mating types of *Paramecium*. Exp. Cell Res. 87: 415-417.

Tsukii Y. (1988) Mating-type inheritance. In: *Paramecium* (Ed. H.-D. Görtz), Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, pp.59-69.

Tsukii Y., Hiwatashi K. (1983) Genes controlling mating-type specificity in *Paramecium caudatum*: Three loci revealed by intersyngenic crosses. Genetics 104: 41-62.

Sonneborn T. M. (1939) Mating types and groups; lethal interactions; determination and inheritance. Am. Naturalist 73: 390-413.

Watanabe T. (1977) Ciliary membranes and mating substances in *Paramecium caudatum*. J. Protozool. 24; 426-429.

Weber K., Osborn M. (1969) The reliability of molecular weight determination of the ciliary ATPase dynein. J. Biol. Chem. 244: 4406-4412.

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Biochemical and Spectroscopic Investigations of the Presumed Photoreceptor for Phototaxis in Amoebae of the Cellular Slime Mold, *Dictyostelium discoideum*

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Summary. In darkness amoebae of the cellular slime mold *Dictyostelium discoideum* (strain AX2) produce a reddish pigment at a higher concentration than when grown in light, the absorption spectrum of which closely resembles the action spectrum for amoebal positive and negative phototaxis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), isoelectric focussing and 2D gel electrophoresis show pronounced differences between light and dark grown cells. Anion exchange column chromatography on a fast protein liquid chromatography (FPLC) system was used to enrich the pigment protein complex. This complex is tightly membrane bound, the protein component has an apparent molecular weight of 45,500 and the chromophoric group has a major maximum at 407 nm plus additional peaks throughout most of the visible spectrum up to 640 nm, which indicates that it might function as the photoreceptor for amoebal phototaxis.

Key words. *Dictyostelium discoideum*, photoreceptor pigment, phototaxis, slime molds.

INTRODUCTION

In the last few decades the cellular slime mold, *Dictyostelium discoideum*, first described by Raper (1935), has developed into a eukaryotic model organism for photobiological, biochemical, developmental and molecular genetic research (Bonner 1982, Gerisch 1982, Kesbeke et al. 1990, Schaap 1986, Knecht and Kessin 1990). In nature the unicellular amoebae occupy the decaying leaf zone in deciduous forests (Cavender and Raper 1965, Cavender 1969, 1973) where they feed on bacteria which they locate using a chemotactic orientation toward folic acid (Pan et al. 1972). The organism has a sexual and an asexual life cycle (Raper 1940, Bonner 1944, Blaskovics and Raper 1957). However, some of the laboratory strains such as the axenic strain AX2 used in this study have lost the sexual cycle altogether.

The vegetative cycle alternates between multicellular pseudoplasmodia, also called slugs because of their morphology, and unicellular (but multinuclear) amoebae. The slugs consist of up to 100,000 individual cells which are not fused as in the case of the acellular slime molds; they move by gliding and orient with respect to external stimuli (Raper 1940, Bonner et al. 1950, Francis 1964). In darkness, the slugs persist in their original direction of movement (Fisher et al. 1983), interrupted however, by spontaneous directional changes (Fisher et al. 1984). In light, slugs orient toward the light source (positive phototaxis). The action spectrum

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of this response significantly differs from that of the amoebae (Poff et al. 1973, 1974) and shows two peaks at 420 and 440 nm and smaller additional maxima up to 610 nm (Poff and Häder 1984). The difference between slug and amoebal phototaxis is further demonstrated by the fact that a slug phototaxis mutant (PT 3) showed a pronounced amoebal phototaxis (Häder et al. 1980). Slugs detect the light direction using a lens effect (Francis 1964, Häder and Burkart 1983): laterally impinging light is focussed onto the distal side since the refractive index of the cytoplasm is sufficiently higher than that of the surrounding air.

In ultraviolet radiation (<200 nm) the lens effect is disturbed by high internal absorption, which causes a negative phototaxis (Häder 1985). This can also be induced experimentally in visible light by incorporating neutral red into the cells which is readily taken up by amoebae before aggregation (Häder and Burkart 1983). The lens effect has also been simulated in an optical model (Poff et al. 1986) and calculated in a mathematical model (Häder and Burkart 1982).

Fisher et al. (1981) have supposed that the focussed light induces the production of a yet unknown, low molecular weight metabolite, termed slug turning factor (STF), which is supposed to act as a chemical repellent. According to this hypothesis, positive phototaxis is the result of negative chemotaxis.

Phototaxis can be bimodal with slugs deviating right or left from the light direction at an angle which depends on the strain and external conditions (Fisher and Williams 1981, Fisher and Williams 1982, Dohrmann et al. 1984, Poff et al. 1986).

When looking for the possible photoreceptor, Poff et al. (1973) found two light-induced absorption changes in vivo as well as in a solubilized mitochondrial extract from amoebae. The action spectra for the absorption change roughly coincided with that of phototaxis (Poff et al. 1973). The photosensitive pigment was isolated by sucrose gradient centrifugation and the molecular weight of the substance calculated to be 200,000 Da by SDS (sodium dodecyl sulfate) electrophoresis (Poff et al. 1974). A light-induced oxidation was thought to be the primary photochemical reaction (Poff and Butler 1974a). In the following report a different pigment system was suggested (Poff and Butler 1974b): a flavin acts as photoreceptor together with a cytochrome b_2, both located on the same protein. Poff et al. (1974) also isolated a photosensitive pigment protein complex with a molecular weight of 200 kDa (seen in gel filtration) or 240 kDa (sedimentation), which they called phototaxin, despite the fact that they assayed a light-induced absorption change in the range of 410 - 430 nm. Also they isolated the pigment from amoebae and interpreted their data in terms of the photoreceptor for slug phototaxis not knowing at that time that the two life stages have different phototaxis action spectra (Häder and Poff 1979a, b, Poff and Häder 1984). Also Poff et al. (1974) could demonstrate a peak at 635 nm only by measuring a difference spectrum between a control and a sample irradiated at very high fluence rates, while the absorption peak at 635 nm is clearly visible in vivo. Thus, the 210 kDa protein cannot be the photoreceptor pigment.

The amoebae also show a pronounced phototaxis which is positive at low light intensities (Hong et al. 1981, Häder and Poff 1979a) and negative at higher (Häder and Poff 1979b). The action spectra of both responses resemble that for photoinhibition of aggregation (Häder and Poff 1979c) and peak at 405 nm and extend throughout most of the visible spectrum up to 640 nm.

Under certain conditions some strains show a bimodal (Häder et al. 1983b) or even multimodal orientation with up to twelve preferred directions (Fisher et al. 1985). Irradiation of single amoebae with low intensity light spots indicated that the mechanism of light direction detection is based on a comparison in the measurements of multiple photoreceptor sites located on the periphery of the cell. High intensity light spots caused pseudopodia to be withdrawn and new ones to be produced in a different part of the cell. In contrast, low light spots induced the development of pseudopodia at the irradiated site (Häder at al. 1983a). These results further indicate that the photoreceptor is located in the cytoplasmic membrane or in the ectoplasm.

In addition to phototaxis, *Dictyostelium* amoebae and slugs show positive and negative thermotaxis with a remarkable sensitivity, e.g., pseudoplasmodia respond to gradients of 0.0005°C across the slug width (corresponding to a gradient of 0.05°C per cm) (Bonner et al. 1950, Poff and Skokut 1977). In both life stages the direction of thermotaxis depends on the change from the growth temperature to the temperature the organisms are exposed to in the gradient (Whitaker and Poff 1980, Fisher and Williams 1982, Hong et al. 1983). Thermotaxis has also been supposed to be mediated by the slug turning factor (STF) (Fisher and Williams 1981). Recently, positive gravitaxis has been shown to occur in Dictyostelium slugs but not in amoebae in the absence of other stronger signals (Häder and Hansel 1991).

The aim of this study is to investigate the photoreceptor for amoebal phototaxis on a molecular level using biochemical and spectroscopic methods.

Abbreviations

CA	carier ampholyte
CAMP	cyclic adenosine monophosphate
EDTA	ethylene diamine tetraacetic acid
Emulphogen	polyoxyethylene 10 tridecyl ether
FPLC	fast protein liquid chromatography
IEF	isoelectric focussing
IPGi	mmobilized pH gradient
NP-40	Nonidet P-40
PMSF	phenyl methyl sulfonyl fluoride
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamid gel electrophoresis
STF	slug turning factor

MATERIALS AND METHODS

Organism and culture conditions

All experiments were carried out with the axenic strain AX2 of *Dictyostelium discoideum* in late logarithmic phase which was grown in Erlenmeyer flasks filled to 40% of their nominal volume with HL5 medium (Ashworth and Watts 1970), with streptomycin added to prevent bacterial contamination. The fresh flasks were inoculated with cells in their late exponential phase (3 - 5 x 10^6 cells ml^{-1}) and kept on a rotary shaker (125 rpm) at 20°C for 3 - 4 days. Once a month amoebae were harvested and allowed to develop spores on phosphate agar plates (2% agar, 14 mM phosphate, pH 6.8). The spores of about 20 sorocarps were then transferred into liquid culture and allowed to germinate.

Spectroscopy

Absorption spectra were recorded in a DU70 single beam spectrophotometer (Beckman, Palo Alto, USA). Cells were harvested at a density of 6 x 10^6 cells ml^{-1} by centrifugation (650 x g, 2MK centrifuge, Sigma, Osterode, FRG) for 10 min, washed in 20 mM phosphate buffer (pH 7.5) and resuspended at a density of 5 x 10^7 cells ml^{-1}. The in vivo spectra were recorded in a 3 ml quartz cuvette (Hellma, Müllheim, FRG); buffer was used as background with several layers of filter paper to compensate for the strong scattering in the sample.

Membrane isolation

Since most photoreceptors are supposed to operate only when bound to a membrane it seemed plausible to isolate the cellular membranes in order to find the photoreceptor. Cells at a concentration of 4 - 8 x 10^6 cells ml^{-1} were harvested at 600 x g, for 10 min at 4°C in a refrigerated centrifuge (J2-21M/E with JA-10 rotor, Beckman). The pellet was resuspended in 5 times the volume of 20 mM phosphate buffer (pH 6.8) and centrifuged again as above and the wash was repeated. The pellet was suspended in a homogenization buffer (20 mM Tris pH 7.5, 1 mM EDTA, 0.1 mM PMSF (phenyl methyl sulfonyl fluoride, 0.1% NaN_3) and sonicated on ice (40% duty cycle, output 5, Branson sonifier 450, Danbury, USA) until 90% of the cells were disrupted (about 4 min). From this homogenate the total membrane fraction was pelleted at 31,300 x g (JA-20, Beckman) at 4°C for 40 min. To remove the remaining cytoplasmic contaminants the membranes were resuspended in ten times their volume and pelleted using the same procedure. The pellet was suspended in 10 mM Tris, 1 mM EDTA, 0.1 mM PMSF, 0.1% NaN_3 and 0.5% Emulphogen (= polyoxyethylene 10 tridecyl ether), sonicated on ice for 1 min (10% duty cycle, output 4) and incubated for 1 h on ice on a stirrer. The resulting extract was centrifuged at 132,000 x g (75Ti rotor, Beckman) in an ultracentrifuge (L8-N, Beckman) for 1 h at 4°C. The supernatant contained the membrane proteins solubilized in 0.5% Emulphogen and the procedure was repeated with the pellet in the same buffer containing 1% Emulphogen to yield soluble proteins in the supernatant and insoluble ones in the pellet.

Gel electrophoresis

SDS polyacrylamid gradient gel electrophoresis was carried out in a vertical system (2001, Pharmacia LKB, Uppsala, Sweden) with gels of 155 x 130 mm, 1.5 mm thick using the method described by Lämmli (1970) with a gradient (5 - 15% T) in the resolving gel. The samples contained 1 mg protein per ml and were diluted with an equal amount of sample buffer.

Isoelectric focussing (IEF, pH 4 - 10 in 4% T polyacrylamide) was performed in the horizontal Multiphor II system (Pharmacia LKB) using an immobilized pH gradient (IPG) rather than carrier ampholyte (CA), the latter of which need a prefocussing and are not so stable over time (Görg et al. 1988). To the sample the same volume of an Ultradex slurry (Pharmacia LKB, 30 mg Ultradex in 1 ml buffer, soaked over night at 4°C) was added to facilitate the sample penetration into the gel and prevent the proteins from precipitating at the pocket edge. The dried IEF gel was cut into strips 5 mm wide and soaked over night; a 20 µl sample was applied and focussing was performed in three steps (150 V and 2 mA, 5 W for 30 min; 300 V at 2 mA, 5 W for 30 min; 3000 V at 2 mA, 5 W for 1 h). After focussing the strips were either stained immediately or used for the second dimension of a 2D gel electrophoresis.

2D gel electrophoresis

2D gel electrophoresis was performed on a horizontal Multiphor II system as described by Görg et al. (1988). The first dimension (IEF) was carried out as described above; however, in order to remove the lipids the homogenate was precipitated in acetone (containing 10% trichloracetic acid and 0.07% (v/v) β-mercaptoethanol) for 45 min at -20°C. The precipitate were pelleted at 42,000 x g (75Ti) for 20 min at -18°C, washed in acetone and resuspended at a concentration of 10 mg per ml in sample buffer. The occurrence of vertical stripes could be largely prevented by adding iodine acetamide in the second equilibration step (Görg et al. 1988, Beis and Lazou 1990).

Native gels were stained with the colloidal Coomassie brilliant blue procedure (Neuhoff 1990) which has the advantage that the large colloidal particles do not penetrate into the gel, but the disperse dye

does and stains the proteins. The silver staining was performed according to Merill et al. (1981). Vertical gels were dried in a gel dryer (model 543, BioRad, Munich, FRG). Gels on GelBondPag films were covered with a cellophane foil soaked with conservation fluid and dried at room temperature.

Gels were scanned using a laser densitometer (Pharmacia LKB) before drying. The evaluation and quantification of proteins was performed with the GelScan-XL software (ver. 2.1, Pharmacia LKB) on an IBM AT computer (model 50, IBM, U.K.).

Fast protein liquid chromatography (FPLC)

Chromatographic separation of the proteins was performed on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB) with contained the following components: control unit (LCC-500 Plus), fraction collector (Frac-100), dual wavelength variable wavelength detector (model 2141, Pharmacia LKB) set to 280 and 407 nm, gradient mixer, sample loop and two motor valves (MV-8 and MV-7). Separation was performed on an ion exchange column Mono Q (HR5/5) using a flow rate of 0.5 ml min^{-1} and an NaCl

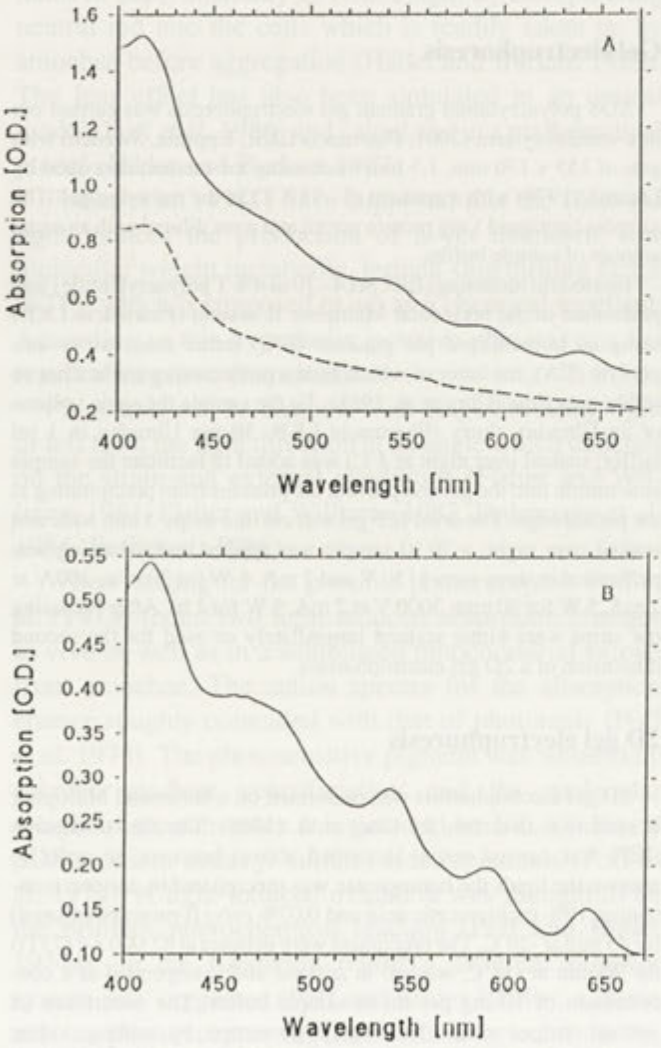

Fig. 1. (A) Absorption spectra of light grown (dashed line) and dark grown (continuous line) *Dictyostelium discoideum* in vivo and (B) difference spectrum between the two

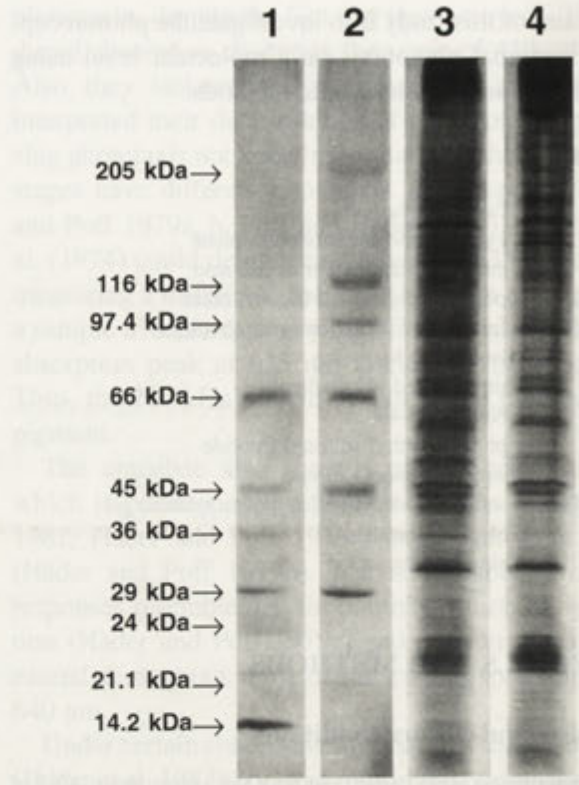

Fig. 2. SDS PAGE of total cell homogenates from dark grown (lane 3) and light grown (lane 4) cells harvested at 4.2 x 10^6 cells per ml in comparison to marker proteins 70L (lane 1) and 6H (lane 2)

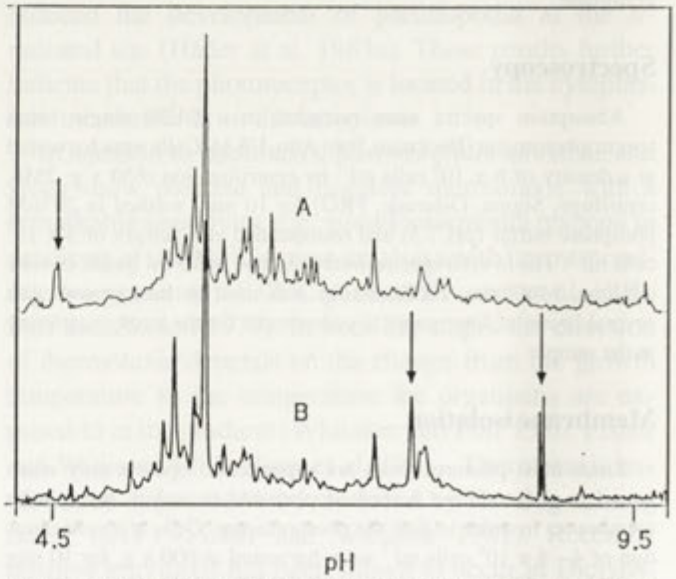

Fig. 3. Gelscans of IEF gels using total cell homogenates of light (A) and dark (B) grown cells. Arrows indicate marked differences between the scans

gradient 0 - 1 M for elution. Programming the runs was done using the FPLC manager software (Pharmacia LKB). All buffers were filtered (0.2 µm pore size, Sartorius, Göttingen, FRG) as were the samples (0.2 µm pore size, Rotrand, Schleicher & Schuell, Dassel, FRG) to remove unsolubilized contaminants. Protein quantification was performed using the method described by Bradford (1976).

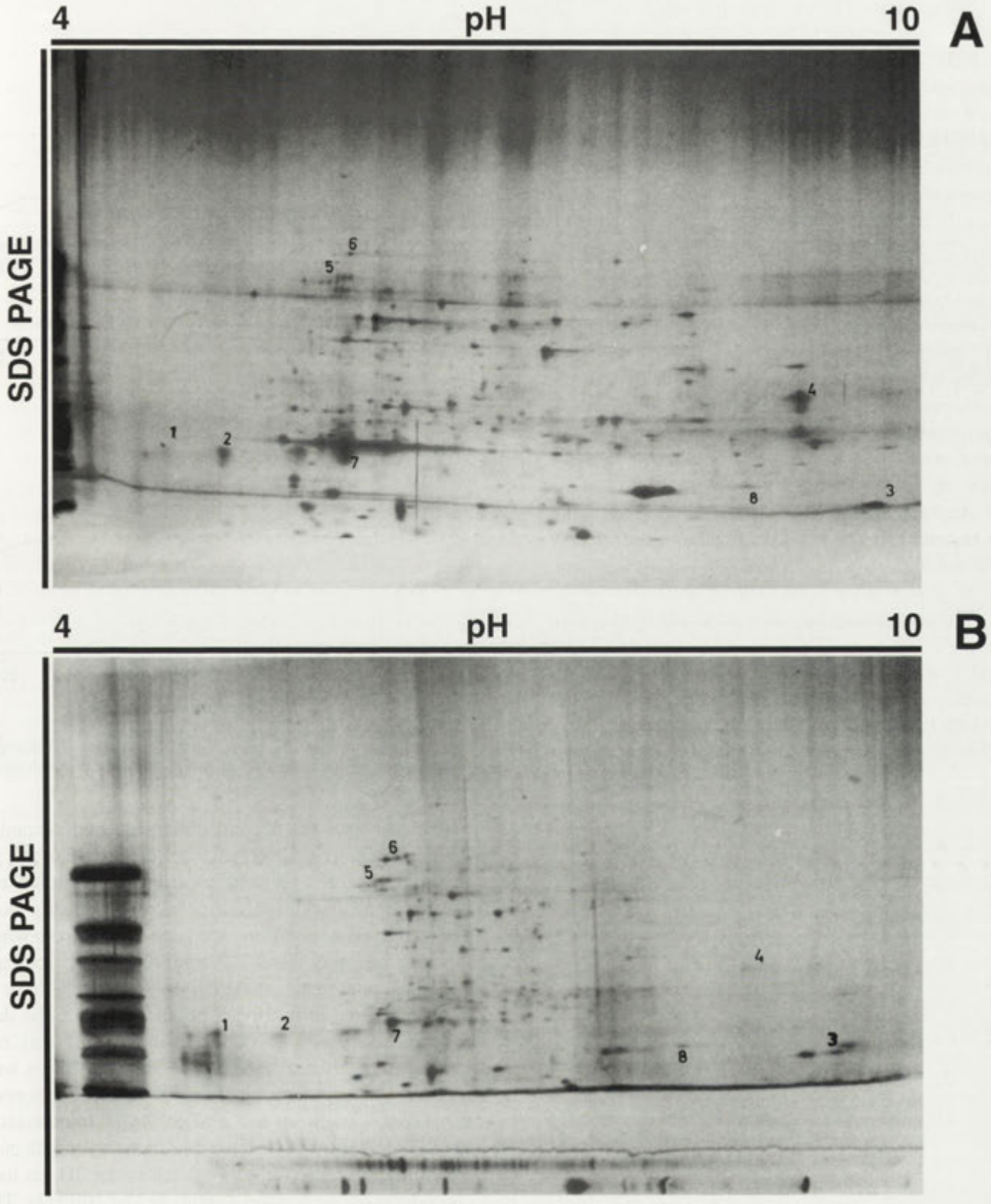

Fig. 4. 2D gel electrophoresis of light (A) and dark (B) grown cell homogenates

RESULTS

When grown in darkness the amoebae of *Dictyostelium discoideum* show a pronounced reddish color, which is not as obvious in cells grown in light. Absorption spectra of dark and light grown cells (at the same cell density) quantitate the effect and a difference spectrum between the two clearly indicates the pigment involved (Fig. 1). This difference absorption spectrum very closely resembles the action spectra of positive and negative phototaxis in amoebae of *Dictyostelium* (Häder and Poff 1979a, b). Basically, the same spectra were obtained for the homogenate prepared for 2D gel electrophoresis (see below, data not shown). Pigment synthesis strongly depends on cell density: cells grown in darkness produce it maximally at a density of 1.2 x 106 cells per ml and light grown ones at 9.7 x 105 cells per ml. In order to investigate the protein composition, cells were homogenized at various densities by sonication and samples were adjusted to 1 mg protein per ml before subjecting them to SDS PAGE (SDS polyacrylamide gel electrophoresis) (Fig. 2). There was a distinct band at 210 kDa apparent molecular weight for the dark grown cells which was almost absent in the light grown culture (data not shown).

The differences between dark and light grown cells was further investigated at the protein level using 2D gel electrophoresis. The initial use of 5 - 6 mm wide isoelectric focussing gels allowed a good focussing but posed two major problems in the second dimension: first, it required very long transition times for protein

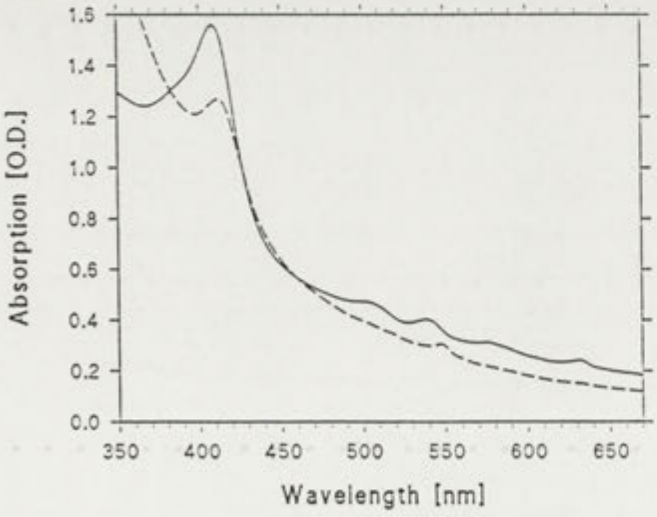

Fig. 5. Absorption spectra of the membrane fraction (solid line) and the cytoplasmic fraction (dashed line)

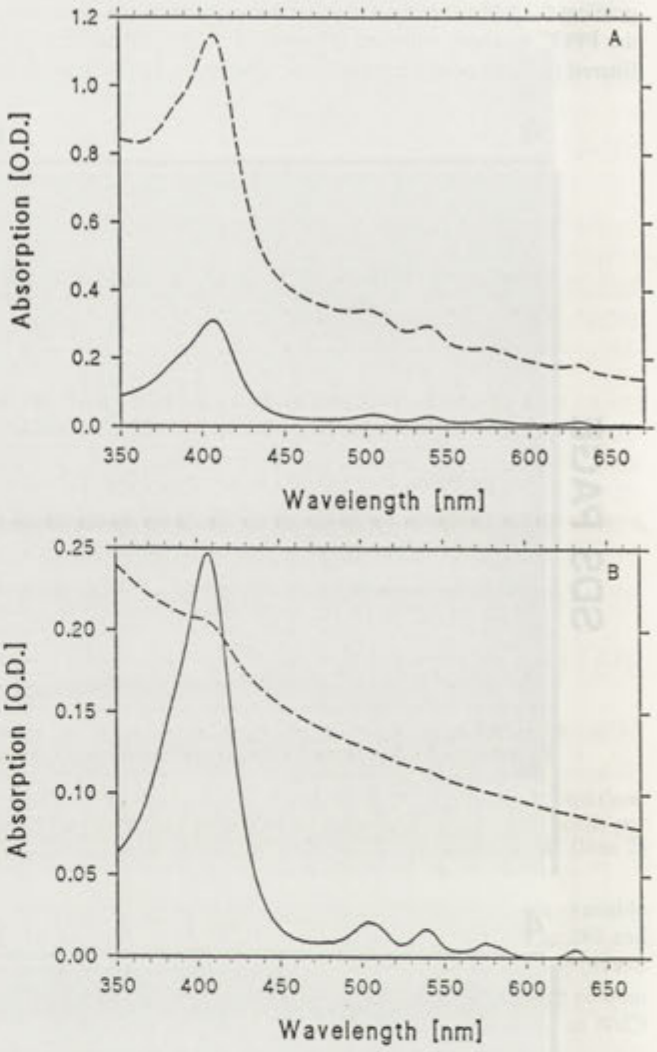

Fig. 6. Absorption spectra of the 0.5% (A) and 1% (B) detergent treatment showing the solubilized (solid line) and non solubilized (dashed line) fractions

penetration into the second dimension and second, a greater amount of detergent was carried into the gel causing the protein spots to smear especially at low pH. Use of 3 mm wide gel strips allowed equally good isoelectric focussing of the total cell extract from which the contaminating lipids had been removed beforehand by an acetone treatment. The light grown cells have an additional band at an IEP of about 4.5, while the dark grown cells show a stronger band at 7.2 and two additional bands near 8.8 (Fig. 3). The IEF strips were used as the first dimension of the 2D gel electrophoresis; the second dimension was a SDS PAGE. In contrast to 1D SDS PAGE, which showed proteins with molecular weights up to 250 kDa, all proteins in the 2D gel have components at molecular weights below 100 kDa. This may be due to the presence of urea in the lysis buffer

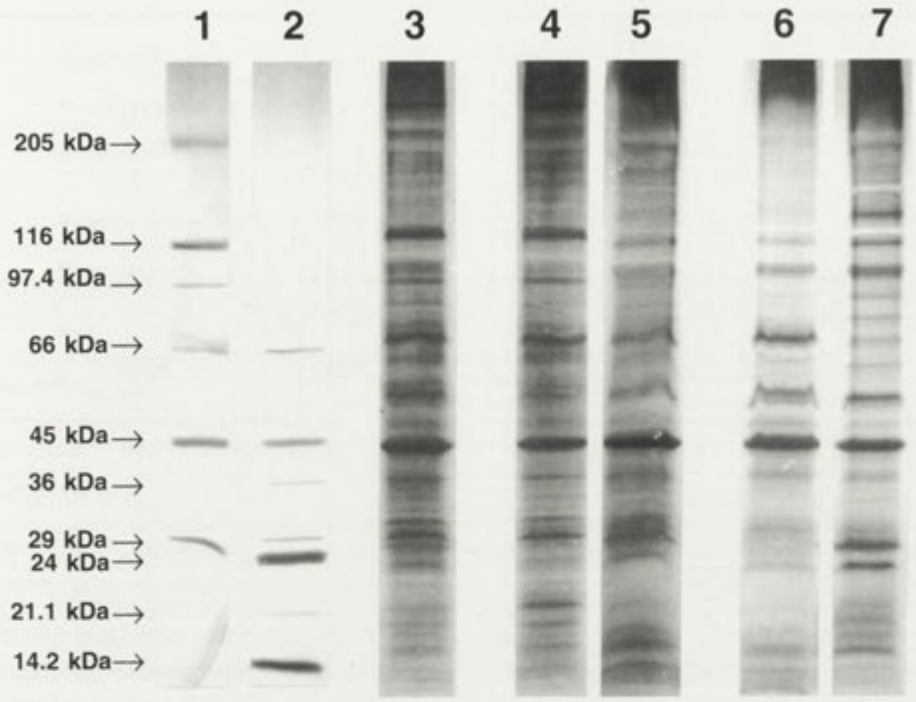

Fig. 7. SDS PAGE of total membrane pellet (lane 3), soluble fraction (lane 4) and pellet (lane 5) in 0.5% detergent and soluble fraction (lane 6) and pellet (lane 7) in 1% detergent in comparison to 6H (lane 1) and 70L (lane 2) marker proteins

which breaks the hydrogen bonds between protein subunits. The 2D gel of the dark grown cells (Fig. 4 A) shows a number of low molecular weight spots in the acid region (1) missing in the light grown cells (Fig. 4 B). In contrast, spot 2 is stronger in the light grown cells; it could represent the peak at pH 4.5 in the IEF gelscan. The two spots (3) are not present in the gel of the light grown cells and seem to represent the double band at pH 8.8. The group of spots (4) in the alkaline region is missing in the dark grown cells. The pattern near (5) is identical in both gels, while two adjacent proteins (6) with relatively high molecular weight are missing in the light grown cells and the obvious spot at (7) is stronger in the light grown cells.

The next set of experiments was designed to purify and characterize the pigment. Absorption spectra of the total membrane fraction and the cytoplasmic fraction from dark grown cells indicate that the majority of the

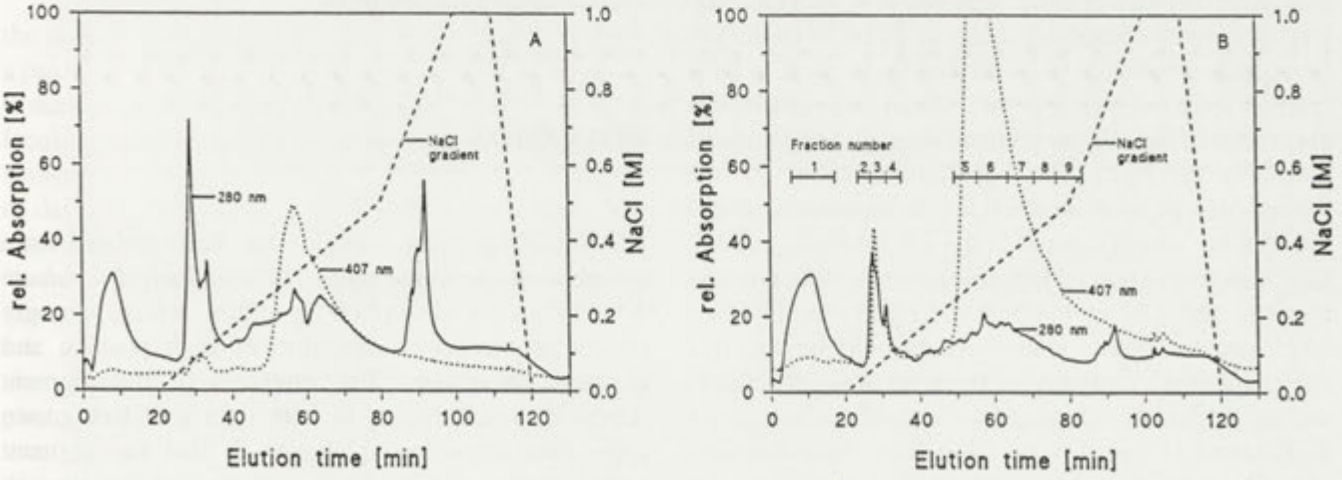

Fig. 8. FPLC elution diagram of the proteins solubilized by (A) 0.5% and (B) 1% detergent at 0.5 ml min^{-1} in a Mono Q ion exchange column. Buffer: 25 mM Tris (pH 9.5, 0.1% (w/v) Emulphogen, 0.1 mM PMSF). 100% relative absorption corresponds to 0.5 O.D. at 280 nm (solid line) and 0.2 O.D. at 407 nm (dotted line), respectively

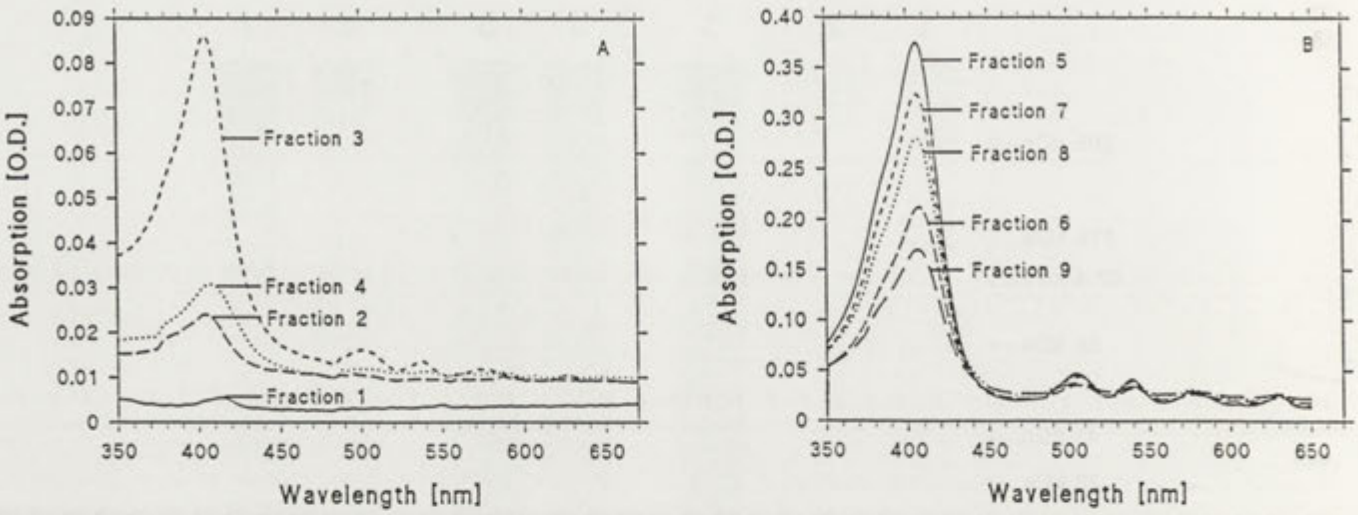

Fig. 9. Absorption spectra of the protein bound chromophore fractions 1 - 4 (A) and fractions 5 - 9 (B) from the FPLC run of the membrane proteins solubilized in 1% detergent

pigment is membrane bound (Fig. 5); it has a maximum at 407 and smaller peaks at 505, 540, 575 and 632 nm. SDS PAGE indicated large differences between the two fractions (data not shown).

The total membrane fraction was solubilized in two steps: at 0.5% detergent both the soluble and the non soluble fraction contained the pigment (Fig. 6 A), however, most of the protein was in the solubilized fraction. By 1% detergent treatment of the non solubilized fraction after centrifugation only the supernatant contained the pigment and the pellet did not and was therefore discarded (Fig. 6 B). SDS PAGE of the four fractions shows a specific enrichment of proteins in some fractions (Fig. 7).

The two soluble fractions were collected and subjected to chromatographic separation in an anion exchange column (Mono Q) using an FPLC system. The first peaks of both the 0.5% and the 1% detergent soluble fraction were not bound to the column and carried little absorption of the 408 nm pigment (Fig. 8). The first peak eluted by the rising NaCl gradient at about 30 min carried little pigment in the 0.5% detergent solubilized material but a high amount in the 1% detergent solubilized material. A very high absorption at 407 nm was found at about 60 min, which did not correspond to a large protein peak, indicating the elution of free chromophores. A number of fractions was collected in the second run as indicated by the numbers in figure 8 B. Absorption spectra were taken from these fractions (Fig. 9). All fractions contained the 407 nm pigment, but in various concentrations; fraction 5 had especially high absorptions.

SDS PAGE of the individual fractions collected from the Mono Q elution were subjected to SDS PAGE. Fractions 2 - 4 showed an enrichment in the 57.3 kDa band, especially fraction 3, which has a slightly lower apparent molecular weight than the main band of fraction 2 (58.8 kDa) (Fig. 10). The remaining fractions had major bands at apparent molecular weights of 45.5 and 74 kDa, as confirmed by gel scans and the comparison with a calibration curve using standard molecular weight proteins. This analysis showed that fractions 2 and 3 contained different proteins (57.3 and 58.8 kDa, respectively), which had already been seen as double band in the 1% detergent solubilized material in SDS PAGE. The 45.5 kDa band in fractions 5 - 9 was also the most prominent in the SDS PAGE of the total non soluble and soluble membrane fractions.

DISCUSSION

A protein pigment complex has been isolated from amoebae of the slime mold *Dictyostelium discoideum* AX2 with an absorption spectrum which strongly resembles the action spectrum of both positive and negative phototaxis. The synthesis of the pigment depends on cell density in both light and dark grown cells. One possible explanation is that the pigment synthesis is controlled by the oxygen concentration in the medium which declines with increasing cell density. Asson-Batres and Hare (1991) found a similar oxygen

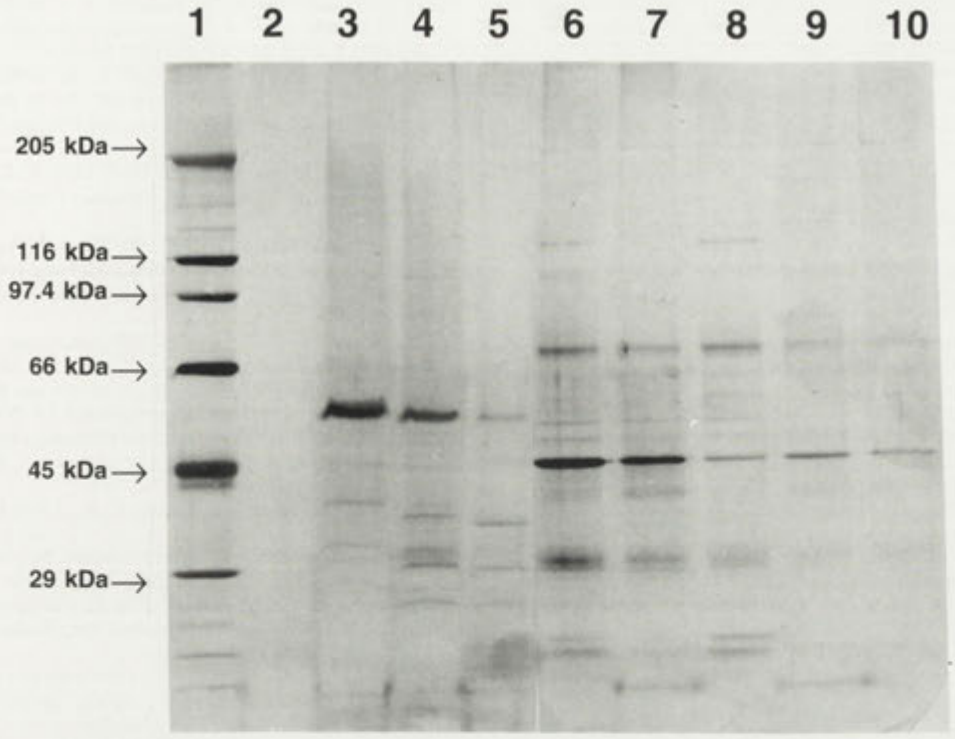

Fig. 10. SDS PAGE of the fractions 1 - 9 (lanes 2 - 10) collected from the FPLC run of the 1% detergent solubilized material in comparison to the 6H marker proteins (lane 1)

dependence of the mitochondrial cytochrome synthesis in yeast. Also the cytochrome c oxidase found in *Dictyostelium discoideum* (Bisson et al. 1985) changes its subunit composition during the transition from logarithmic to stationary growth (Bisson and Schiavo 1986) which is also due to a change in the oxygen concentration in the medium (Schiavo and Bisson 1989).

The more important external factor for this study is the dependence of synthesis on light: the SDS gel shows a peak at an apparent molecular weight of about 210 kDa in dark grown cells which is lacking in light grown cells. Isoelectric focussing and 2D gel electrophoresis also showed a number of differences in the protein pattern of dark and light grown cells. In the membrane fraction a protein could be enriched with the matching absorption characteristics. Already the first centrifugation had removed a cytochrome fraction which is either cytoplasmic or only loosely bound to the membrane. Their absorption characteristics disqualify them as photoreceptor pigments (Manabe and Poff 1978). Since most of the pigment could be solubilized only by 1% detergent treatment the receptor seems to be tightly bound to the membrane. Ion exchange chromatography separated two components with a 407 nm absorption maximum, the molecular weights of which were subsequently shown by SDS PAGE to be 57.3 and 45.5 kDa, respectively. The latter band changed in intensity during growth which qualifies this protein to carry the photoreceptor chromophore.

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REFERENCES

Ashworth J. M., Watts D. J. (1970) Metabolism of the cellular slime mold *Dictyostelium discoideum* in axenic culture. Biochem. J. 119: 175-182.

Asson-Batres M. A., Hare J. F. (1991) Effect of oxygen on the synthesis and assembly of mitochondrial encoded subunits of cytochrome oxidase and cytochrome b, c_1 in mouse embryo fibroblasts. J. Biol. Chem. 266: 9932-9938.

Beis A., Lazou A. (1990) Removal of artifactural bands associated with the presence of 2-mercaptoethanol in two-dimensional polyacrylamid gel electrophoresis. Anal. Biochem. 190: 57-59.

Bisson R., Schiavo G. (1986) Two different forms of cytochrome c oxidase can be purified from the slime mold *Dictyostelium discoideum*. J. Biol. Chem. 261: 4373-4376.

Bisson R., Schiavo G., Papini E. (1985) Cytochrome c oxidase from the slime mold *Dictyostelium discoideum*: purification and characterization. Biochemistry 24: 7845-7852.

Blaskovics J. A., Raper K. B. (1957) Encystment stages of *Dictyostelium*. Biol. Bull. 113: 58-88.

Bonner J. T. (1944) A descriptive study of the development of the slime mold *Dictyostelium discoideum*. Amer. J. Bot. 31: 175-182.

Bonner J. T. (1982) Comparative biology of cellular slime molds. In: Developmental Biology of *Dictyostelium* (Ed. W. F. Loomis), pp. 1-33. Academic Press, New York.

Bonner J. T., Clarke, W. W. Jr., Neely C. L. Jr., Slifkin M. K. (1950) The orientation to light and the extremely sensitive orientation to temperature gradients in the slime mold *Dictyostelium discoideum*. J. Cell Comp. Physiol. 36: 149-158.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cavender J. C. (1969) The occurrence and distribution of Acrasieae in forest soils. I. Europe. Amer. J. Bot. 56: 9:989-992.

Cavender J. C. (1973) Geographical distribution of Acrasiae. Mycologia 65: 1044-1054.

Cavender J. C., Raper K. B. (1965) The Acrasieae in nature. II. Forest soil as a primary habitat. Amer. J. Bot. 52: 297-302.

Dohrmann U., Fisher P. R. (1984) Transitions in *Dictyostelium discoideum* behaviour: influence of calcium and fluoride on slug phototaxis and thermotaxis. J. Cell Sci. 65: 111-121.

Fisher P. R., Dohrmann U., Williams K. L. (1984) Signal processing in *Dictyostelium discoideum* slugs. Mod. Cell Biol. 3: 197-248.

Fisher P. R., Grant W. N., Dohrmann U., Williams K. L. (1983) Spontaneous turning behaviour by *Dictyostelium discoideum* slugs. J. Cell Sci. 62: 161-170.

Fisher P. R., Häder D.-P., Williams K. L. (1985) Multidirectional phototaxis by *Dictyostelium discoideum* amoebae. FEMS Microbiol. Lett. 29: 43-47.

Fisher P. R., Smith E., Williams K. L. (1981) An extracellular chemical signal controlling phototactic behaviour by *Dictyostelium discoideum* slugs. Cell 23: 799-807.

Fisher P. R., Williams K. L. (1981) Bidirectional phototaxis by *Dictyostelium discoideum* slugs. FEMS Microbiol. Lett. 12: 87-89.

Fisher P. R., Williams K. L. (1982) Thermotactic behaviour of *Dictyostelium discoideum* slug phototaxis mutants. J. Gen. Microbiol. 128: 965-971.

Francis D. W. (1964) Some studies on phototaxis of *Dictyostelium*. J. Cell Comp. Physiol. 64: 131-138.

Gerisch G. (1982) Chemotaxis in *Dictyostelium*. Ann. Rev. Physiol. 44: 535-552.

Görg A., Postel W., Günther S. (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9: 531-546.

Häder D.-P. (1983) Effects of UV-B irradiation on sorocarp development of *Dictyostelium discoideum*. Photochem. Photobiol. 38: 551-555.

Häder D.-P. (1985) Negative phototaxis of *Dictyostelium discoideum* pseudoplasmodia in UV radiation. Photochem. Photobiol. 41: 225-228.

Häder D.-P., Burkart U. (1983) Optical properties of *Dictyostelium discoideum* pseudoplasmodia responsible for phototactic orientation. Exp. Mycol. 7: 1-8.

Häder D.-P., Claviez M., Merkl R., Gerisch G. (1983a) Responses of *Dictyostelium discoideum* amoebae to local Stimulation by Light. Cell Biol. Internatl. Rep. 7: 611-615.

Häder D.-P., Hansel A. (1991) Responses of *Dictyostelium discoideum* to multiple environmental stimuli. Bot. Acta 104: 200-205.

Häder D.-P., Poff K. L. (1979a) Light-induced accumulation of *Dictyostelium discoideum* amoebae. Photochem. Photobiol. 29: 1157-1161.

Häder D.-P., Poff K. L. (1979b) Photodispersal from light traps by amoebae of *Dictyostelium discoideum*. Exp. Mycol. 3: 121-131.

Häder D.-P., Poff K. L. (1979c) Inhibition of aggregation by light in the cellular slime mold *Dictyostelium discoideum*. Arch. Microbiol. 123: 281-285.

Häder D.-P., Whitaker B. D., Poff K. L. (1980) Responses to light by a nonphototactic mutant of *Dictyostelium discoideum*. Exp. Mycol. 4: 382-385.

Häder D.-P., Williams K. L., Fisher P. R. (1983b) Phototactic orientation by amoebae of *Dictyostelium discoideum* slug phototaxis mutants. J. Gen. Microbiol. 129: 1617-1621.

Hong C. B., Fontana D. R., Poff K. L. (1983) Thermotaxis of *Dictyostelium discoideum* amoebae and its possible role in pseudoplasmodial thermotaxis. Proc. Natl. Acad. Sci. USA 80: 5646-5649.

Hong C. B., Häder M. A., Häder D.-P. Poff K. L. (1981) Phototaxis in *Dictyostelium discoideum* amoebae. Photochem. Photobiol. 33: 373-377.

Kesbeke F., van Haastert P. J. M., de Wit R. J. W., Snaar-Jagalska B. E. (1990) Chemotaxis to cyclic AMP and folic acid is mediated by different G-proteins in *Dictyostelium discoideum*. J. Cell Sci. 96: 669-673.

Knecht D. A., Kessin R. H. (1990) Recent advances in the molecular genetics of *Dictyostelium*. Dev. Genet. 11: 388-390.

Lämmli U. K., (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227: 680-685.

Manabe K., Poff K. L. (1978) Purification and characterization of the photoreducible b-type cytochrome from *Dictyostelium discoideum*. Plant Physiol. 61: 961-966.

Merill C. R., Goldman D., Sedman S. A., Ebert H. (1981) Ultrasensitive stain for proteins in polyacrylamid gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1437-1438.

Neuhoff V. (1990) Die kolloidale Coomassie-Blau-Färbung von Proteinen und Peptiden in Polyacrylamidgelen: Schnell, untergrundfrei und mit Nanogramm-Empfindlichkeit. Biol. Chem. Hoppe-Seyler 371: 10-11.

Pan P., Hall E., Bonner J. T. (1972) Folic acid as a second chemotactic substance in the cellular slime molds. Nature 237: 181-182.

Poff K. L., Butler W. L. (1974a) Absorbance changes induced by blue light in *Phycomyces blakesleeanus* and *Dictyostelium discoideum*. Nature 248: 799-801.

Poff K. L., Butler W. L. (1974b) Spectral characteristics of the photoreceptor pigment of phototaxis in *Dictyostelium discoideum*. Photochem. Photobiol. 20: 241-244.

Poff K. L., Butler W. L., Loomis W. F. (1973) Light-induced absorbance changes associated with phototaxis in *Dictyostelium*. Proc. Natl. Acad. Sci. USA 70: 813-816.

Poff K. L., Fontana D. R., Häder D.-P., Schneider M. J. (1986) An optical model for phototactic orientation in *Dictyostelium discoideum* slugs. Plant Cell Physiol. 27: 533-539.

Poff K. L., Häder D.-P. (1984) An action spectrum for phototaxis by pseudoplasmodia of *Dictyostelium discoideum*. Photochem. Photobiol. 39: 433-436.

Poff K. L., Loomis W. F., Butler W. L. (1974) Isolation and purification of the photoreceptor pigment associated with phototaxis in *Dictyostelium discoideum*. J. Biol. Chem. 249 2164-2167.

Poff K. L., Loomis W. F. jr. (1973) Control of phototactic migration in *Dictyostelium discoideum*. Exp. Cell Res. 82: 236-240.

Poff K. L., Skokut M. (1977) Thermotaxis by pseudoplasmodia of *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA 74: 2007-2010.

Raper K. B. (1935) *Dictyostelium discoideum*, a new species of slime mold from decaying forest leaves. J. Agric. Res. 50: 135-147.

Raper K. B. (1940) Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. J. Elisha Mitchell Sci. Soc. 56: 241-282.

Schaap P. (1986) Regulation of size and pattern in the cellular slime molds. Differentiation 33: 1-16.

Schiavo G., Bisson R. (1988) Oxygen influences the subunit structure of cytochrome c oxidase in the slime mold *Dictyostelium discoideum*. J. Biol. Chem. 264: 7129-7134.

Whitaker B. D., Poff K. L. (1980) Thermal adaption of thermosensing and negative thermotaxis in *Dictyostelium*. Exp. Cell Res. 128:87-93.

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Revision of the Genus *Odonaticola* Sarkar et Haldar, 1981 (Apicomplexa: Sporozoasida: Gregarinasina)

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Summary. Based on literature, four species of gregarine parasites of odonate insects, originally placed in the genus *Hoplorhynchus* Carus, are transferred to the genus *Odonaticola* Sarkar et Haldar. The following new combinations are proposed: *O. gracilis* nov. comb. (Hoshide, 1953); *O. magnus* nov. comb. (Hoshide, 1958); *O. polyhamatgus* nov. comb. (Hoshide, 1977); and *O. orthetri* nov. comb. (Hoshide, 1953). Since the latter species represents a junior secondary homonym to *O. orthetri* Sarkar et Haldar, 1981, a replacement name *O. hoshidei* Amoji et Kori nov. nom. is proposed.

Key words: Genus *Odonaticola*, revision.

The subfamily *Menosporinae* Leger, 1982 as emended by Grasse, 1953, (Family Actinocephalidae Leger, 1892) is characterised by:

1. Epimerite cup-like; with marginal hooks; neck long.
2. Oocysts smooth, crescentic.

Two genera have been placed in this subfamily- *Menospora* Leger, 1892 and *Hoplorhynchus* Carus, 1863.

Recently, a third genus *Odonaticola*, was created and placed in the subfamily by Sarkar et Haldar, 1981 with the following diagnostic features:

1. Epimerite hat-shaped; marginal spines petaloid; neck long.
2. Sporonts solitary.
3. Gametocysts dehisce by simple rupture.
4. Oocysts smooth. boat-shaped.
5. Development extracellular.

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Sarkar and Haldar (1981) described four species under this genus, namely, *Odonaticola longicollera, O. orthetri, O. hexacantha* and *O. rodgii*. During the same year Sarkar (1981) described another species, *O. elliptica* and in addition transferred the gregarine, *Menospora nonacantha*, described by Devdhar and Deshapande (1971) to the genus *Odonaticola*. Kori and Amoji (1983, 1984) reported on three more species *(O. crocothemis, O. haldari* and *O. diplacodi)* which they placed in this genus.

A review of the literature on cephaline gregarines of odonate insects revealed that several species in the genus *Hoplorhynchus* Carus reported (Hoshide 1953, 1958 and K. Hoshide 1977) with complete life stages possess features characteristic of the genus *Odonaticola* Sarkar et Haldar. The genus *Hoplorhynchus* was considered by Kamm (1922) to be characterised by the following features:

1. Epimerite fat disk-like with 8-10 short digitiform processes at the apex of a long neck.
2. Oocysts biconical.

Table 1

Comparison of characters of genera *Hoplorhyncus* Carus, 1863 and *Odonaticola* Sarkar et Haldar, 1981

Genus	Character	
	Epimerite	Oocysts
Hoplorhyncus	Flat disc with 8-10 digitiform process	Crescentic
Odonaticola	Hat-shaped with varying number of petaloid spines at the margin	Boat-shaped

Later Grasse (1953) and Chakravarty (1959), while revising the generic features stated that the oocysts of *Hoplorhynchus* were crescentic and smooth. Hence the taxonomic placement of some of the gregarine species reported under the genus *Hoplorhynchus* needs to be reconsidered.

H. Hoshide (1953, 1958) and K. Hoshide (1977) described new species which they placed in the genus *Hoplorhynchus*, namely *H. gracilis, H. magnus, H. polyhematus* and *H. orthetri*. Although they assumed that the structure of the epimerite and oocysts resembled those of that genus, in actual fact they do not agree with the generic characters stated by Grasse (1953) and Chakravarty (1959), (see Table 1). Illustrations provided by the authors clearly indicate that individual oocysts are boat-shaped and aggregated forms in three or four are triangular or tetrahedral in shape respectively. None are crescentic in shape. Moreover these illustrations reveal an umbrella or hat-shaped epimerite bordered with petaloid spines, but never a flat disc with digitiform processes. As the shape of the epimerite and oocysts of the above mentioned four gregarines closely resemble the features of the genus *Odonaticola* they are herein shifted from the genus *Hoplorhynchus* Carus, 1863 to the genus *Odonaticola* Sarkar et Haldar, 1981 as follows:

Odonaticola gracilis (Hoshide, 1953) nov. comb.
Odonaticola magnus (Hoshide, 1958) nov. comb.
Odonaticola polyhematus (Hoshide, 1977) nov. comb.
Odonaticola orthetri (Hoshide, 1953) nov. comb.

The new combination *O. orthetri* (Hoshide, 1953) becomes a homonym since Sarkar and Haldar (1981) have already described a gregarine with the same name (*O. orthetri* Sarkar et Halder, 1981). According to Articles 52, 57 and 60 of the International Code of Zoological Nomenclature (1985, Third Edition), the proposed new combination *O. orthetri* (Hoshide, 1953)

Table 2

Alphabetical list of species of gregarine parasites currently recognized in the genus *Odonaticola* Sarkar et Haldar incorporating changes as indicated in present paper

Parasite species	Host species
O. crocothemis Kori & Amoji, 1983	*Crocothemis servilia* (Drury)
O. diplacodi Kori & Amoji, 1986	*Diplacodes trivialis* (Ramb.)
O. elliptica Sarkar, 1981	*Crocothemis servilia* (Drury)
O. gracilis (H. Hoshide, 1953) nov. comb.	*Aciagrion hisopa* Selys
O. haldari Kori & Amoji, 1984	*Trithemis aurora* (Burmeister)
*O. hexacantha** Sarkar & Haldar, 1981	*Brachythemis contaminata* (Febr.)
O. hoshidei Amoji & Kori nov. nom. [*Hoplorhynchus orthetri* (Hoshide, 1953)] [*O. orthetri* (Hoshide, 1953) nov. comb.]	Orthetrum albistylum speciosum Uhler
O. longicollera Sarkar & Haldar, 1981	*Diplacodes trivilasis* (Rambur)
O. magnus (Hoshide, 1953) nov. comb. [*Hoplorhynchus magnus*]	*C. servilia* (Drury)
O. nonacantha (Devdhar & Deshapande, 1971)	*Urothemis S. signata*
O. orthetri Sarkar & Haldar, 1981	*Orthetrum sabina* (Drury)
O. polyhematus (K. Hoshide, 1977) nov. comb. [*Hoplorhynchus polyhematus*]	*Munais strigata* Selys
O. rodgii Sarkar & Haldar, 1981	*Neurothermis tullia* (Drury)

* type species for genus *Odonaticola*

becomes a junior secondary homonym. As per the law of homonymy the name of the junior homonym is rejected and replaced by *Odonaticola hoshidei* Amoji et Kori nov. nom.

At present the genus *Odonaticola* contains 13 species as summarized in Table 2.

REFERENCES

Chakravarty M. (1959) Systematic position of some genera and classification of the Suborder Cephalina Delage and Herouard. Proc. Zool. Soc. Calcutta 12: 71-81.

Devdhar M.J., Deshapande S.B. (1971) On two new gregarines of the genus Gregarina from *Mesomorphus velliger* Blanc (Coleoptera: Tenebrionidae) from Dhawar, India. Ind. Zool. 2: 27-34.

Grasse P.P. (1953) Classe des Gregarinomorphes. In: Traite de Zoologie 1, (Ed.P.P.Grasse). Mason, Paris, 550-690.

Hoshide H. (1953) Studies on gregarines parasitic in Japanese insects. VI. Zool. Mag. Tokyo, 62: 166-169.

Hoshide H. (1958) Studies on the cephaline gregarines of Japan II. Bull. Fac. Educ. Yamaguchi Univ. 8: 35-40.

Hoshide K. (1977) Notes on the gregarines in Japan. VIII. Three new species of eugregarines from *Odonata*. Bull. Fac. Educ. Yamaguchi Univ. 27: 93-125.

Kori S.S., Amoji S.D. (1983) On the life history of a new actinocephalid gregarine, *Odonaticola crocothemis* sp. nov. from the gut of an odonate insect, *Crocothemis servilia* (Drury). Ind. Zool. 7: 155-158.

Kori S.S., Amoji S.D. (1984) A new actinocephalid gregarine, *Odonaticola haldari* n. sp. from odonate insect *Trithemis aurora* (Burm.). Acta Protozool. 23: 63-66.

Kori S.S., Amoji S.D. (1986) On an actinocephalid gregarine (Apicomplexa: Sporozoea) *Odonaticola diplacodi* sp. nov. from odonate insect, Diplacodes trivialis (Rambur). Arch. Protistenk. 131: 165-169.

Sarkar N.K. (1981) On some actinocephalid gregarines (Apicomplexa: Eugregarinda) of odonate insects from West Bengal, India. Proc. Ind. Acad. Sci. 90: 649-657.

Sarkar N.K., Haldar D.P. (1981) Observations on four new species of actinocephalid gregarines (Protozoa: Sporozoea) under a new genus *Odonaticola* from odonate insects. Arch. Protistenk. 124: 288-302.

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ACTA
PROTOZOOLOGICA

The Coccidian Parasites (Apicomplexa: Coccidia) of the Herbivorous Mole-rat *Spalax (Microspalax) ehrenbergi* Nehring (Rodentia: Spalacidae) from Syria

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Summary. Four species of *Eimeria* and one of *Isospora* are reported from herbivorous mole-rat *Spalax (Microspalax) ehrenbergi* from Syria. A new species, *Eimeria microspalacis* n. sp., has been described. The coccidian found, according to the frequency of their prevalence, may be arranged in the following way: *E. spalacis* (72%), *E. oytuni* (48%), *E. celebii* (41%), *E. microspalacis* n. sp. (24%) and *Isospora anatolicum* (21%). All the 29 animals examined were infected by coccidian. *Spalax (Microspalax) ehrenbergi* is pointed as a new host of *I. anatolicum*.

Key words. Coccidia, *Eimeria, Isospora,* herbivorous mole-rat, *Spalax (Microspalax) ehrenbergi.*

INTRODUCTION

The coccidian fauna of rodents of the family Spalacidae is explored insufficiently because of their hidden underground way of life and the difficulties encountered in collecting the animals. Only three publications concerning the coccidian of *Spalax (Mesospalax) leucodon* Nordmann and *S. (Microspalax) ehrenbergi* Nehring are known so far. In the two hosts the total of 16 different species of coccidian are described, 15 of which belong to the genus *Eimeria* and one to *Isospora* (Veissov 1975, Sayin et al. 1977, Sayin 1980).

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MATERIALS AND METHODS

29 animals of *Spalax (Microspalax) ehrenbergi* Nehring were collected in the years 1989-1991 in 3 regions in Western Syria: Daraa, Damascus and Latakia. After the dissection of the animal part of its small intestine the content was taken and preserved for laboratory research in a 2,5 % solution of $K_2Cr_2O_7$. A microscopical examination was made after flotation in a saturated NaCl solution by the Fülleborn method. For studying the oocyst sporulation time some positive samples were incubated in a thermostat at 24 - 25°C. They were macerated in distilled water, placed in Petri dishes and covered by humid filter paper. The observations and photographs were made by a microscope NU-2 (Zeiss, Jena) with magnification from 100 to 400 x. The variation of the oocyst size was calculated according to Darwish and Golemansky (1991).

The abbreviations used in the text and in the tables are: m - standard error; L - length of the oocysts; W - width of the oocysts; M - the mean and Lim - limits of the variations.

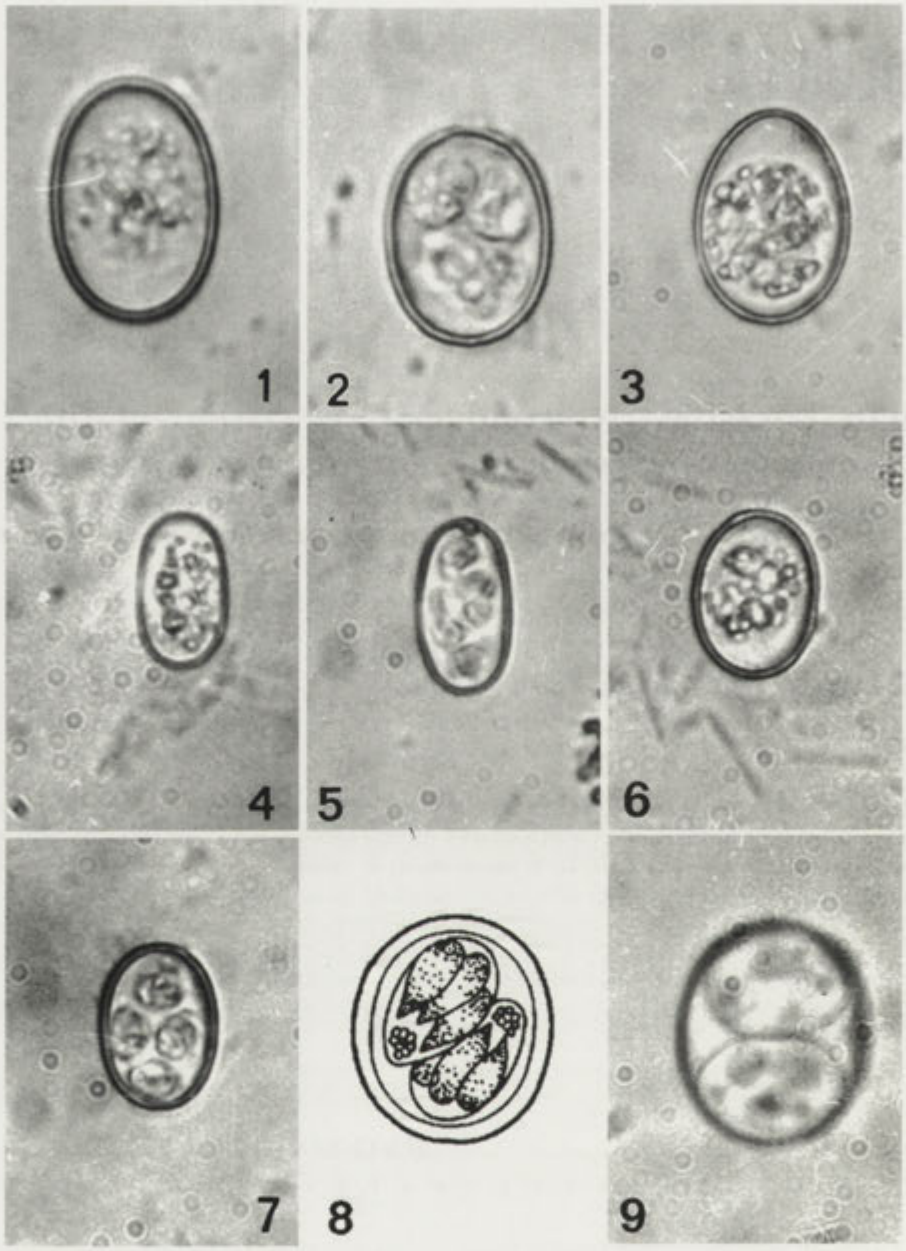

Fig. 1. and 2. *E. microspalacis* n. sp.; 1 - unsporulated oocyst (x 1500); 2 - sporulated oocyst (x 1400). Fig. 3. *E. oytuni* Sayin (x 1200). Fig. 4. and 5. *E. spalacis* Sayin, Dincer et. Meric; 4 - unsporulated oocyst; 5 - sporulated oocyst (x 1000). Fig. 6. and 7. *E. celebii* Sayin; 6 - unsporulated oocyst; 7 - sporulated oocyst (x 1400). Fig. 8. and 9. *I. anatolicum* Sayin, Dincer et. Meric; 8 - sporulated oocyst (graphic) (x 2800); 9 - sporulated oocyst (x 2350)

RESULTS

In the explored herbivorous mole-rat *S. (M.) ehrenbergi* from Syria the following 5 coccidian species were identified:

Eimeria microspalacis n. sp. Plate, fig. 1, 2.

Oocyst morphometry: The oocysts are ellipsoidal, widely oval or rarely subspherical. The oocyst wall is yellowish-brown, smooth and about 1.25 - 1.5 µm thick. Micropyle is absent. The cytoplasm of unsporulated oocysts is more or less granular. In the sporulated oocysts the residuum is absent, but from time to time one polar granule is observed. The oocyst dimensions are presented in Table 1.

The sporocysts are widely ellipsoidal, with a clear Stieda body. Their measurements vary from 7.5-10 by 5-7 µm. (M: 8.8 x 5.8 µm). The sporocyst residuum is compact and composed of large coarse granules.

Table 1

Eimeria microspalacis n. sp.: oocyst dimensions and their variations

	$\bar{x}$ + m	L/W	S.D.	C.V.	Lim	n
L:	20.1 + 0.22	1.12 - 1.5	1.51	7.5	17.5 - 22.5	47
W:	15.38 + 0.14	M = 1.3	0.93	6.04	13.5 - 18.0	47

Prevalence: *E. microspalacis* n. sp. was found in 7 of the 29 examined animals (24.1%).

Localities: The regions of Damascus and Latakia.

Discussion: Five of the seven species of *Eimeria* described so far from *S. (M.) ehrenbergi* in South-Eastern Turkey (*E. urfensis, E. adiyamanensis, E. haranica, E. marasensis* and *E. oytuni*) are characteristic through their clearly noticeable micropyle on one end of the oocyst. Owing to this basic taxonomic character they differ considerably from *E. microspalacis* n. sp. The taxa which we have described can easily be differentiated from the other two species (*E. celebii* and *E. torosicum*) mainly by their form and higher oocyst dimensions. The oocysts of *E. microspalacis* n. sp. are quite similar to those of *E. maralikiens* Veissov, 1975, a parasite in *Spalax leucodon* from Armenia. But geographic the isolation of the two hosts and their different specific status, as well as the strict specificity of the rodent *Eimeria*, constitute a considerable reason to accept *E. microspalacis* n. sp. as a new, specific parasite of *S. (M.) ehrenbergi* inhabiting the Middle Orient.

Eimeria oytuni Sayin, 1980. Plate, fig. 3

Oocyst morphometry: the oocysts are egg-shaped, yellowish-brown, with a smooth oocyst wall, 1-1.5 µm thick. Micropyle is lacking, but sometimes the wall of the narrow end of the oocyst seems thinner and resembles a micropyle.The oocyst residuum and polar granule are absent. The oocyst measurements are presented in Table 2.

The sporocysts are pear-shaped, with a small Stieda body. The sporocyst residuum is composed of small granules. Their dimensions vary from 9-12.5 µm in length to 6-9 µm in width.

Localities: the regions of Daraa, Damascus and Latakia.

Prevalence: *E. oytuni* was found in 14 of 29 examined animals (48.3%).

Eimeria spalacis Sayin, Dincer et Meric, 1977. Plate, fig. 4, 5.

Oocyst morphometry: The oocysts are cylindrical to ellipsoidal and colourless. The oocyst wall is smooth and composed of one layer, about 1 µm thick. Micropyle is absent. The oocyst residuum is lacking but one polar granule is always present. The oocyst measurements are presented in Table 3.

Table 2

Eimeria oytuni: oocyst dimensions and their variations

	$\bar{x}$ + m	L/W	S.D.	C.V.	Lim	n
L:	24.73 + 0.3	1.11 - 1.47	2.13	8.75	18.75 - 28	52
W:	19.16 + 0.14	M = 1.26	1.86	9.7	16.24 - 23.73	52

The sporocysts are ovoid with a sporocyst residuum and a Stieda body, 9-10 x 6-7.5 µm (M = 9.6 x 6.8 µm).

Localities: The regions of Daraa, Damascus and Latakia.

Prevalence: *E. spalacis* was found in 21 of 29 examined animals (72.4%).

Discussion: *E. spalacis* was found and originally described by Sayin, Dincer and Meric (1977) in *Spalax (Mesospalax) leucodon* from the region of Ankara, Central Turkey. In fact, the dimensions of the oocysts, which we observed in *S. (M.) ehrenbergi* (M = 19,38 x 11.91 µm), are a little bigger than those from *S. (M.) leucodon* (M = 16.3 x 9.8 µm), but all the other morphological data and the ratio L/W are comparable. For the moment, we cannot find any considerable morphological differences between the oocysts from the two examined hosts and we suppose that *E. spalacis* is a common parasite of both rodent species.

Eimeria celebii Sayin, 1980. Plate, fig. 6, 7.

Oocyst morphometry: The oocysts are subspherical to ellipsoidal, the oocyst wall is smooth, yellowish, about 1 µm thick. Micropyle and the oocyst residuum are absent, sometimes one polar granule is present. Our measurements and their variations are presented in Table 4.

The sporocysts are ovoid, 5 - 8.25 by 3.75 - 5.5 µm, with a small Stieda body.

Localities: The regions of Daraa and Latakia.

Prevalence: *E. celebii* was found in 12 of 29 examined animals (41.4%).

Table 3

Eimeria spalacis: oocyst dimensions and their variations

	$\bar{x}$ + m	L/W	S.D.	C.V.	Lim	n
L:	19.38 + 0.17	1.3 - 2.25	1.83	9.44	15 - 23	120
W:	11.91 + 0.11	M = 1.7	1.24	10.24	10 - 15	120

Table 4

Eimeria celebii: oocyst dimensions and their variations

	$\bar{x}$ + m	L/W	S.D.	C.V.	Lim	n
L:	14.45 + 0.17	1.1 - 1.62	0.97	6.71	12.5 - 16.25	34
W:	11.31 + 0.23	M = 1.28	1.32	11.67	8.75 - 13.0	34

Table 5

Eimeria anatolicum: oocyst dimensions and their variations

	$\bar{x}$ + m	L/W	S.D.	C.V.	Lim	n
L:	11.87 + 0.2	1.0 - 1.5	1.02	8.59	10 - 15	26
W:	9.98 + 0.17	M = 1.19	0.87	8.71	8.75 - 11.75	26

Isospora anatolicum Sayin, Dincer et Meric, 1977. Plate, fig. 8, 9.

Oocyst morphometry: The oocysts are spherical or subspherical, sometimes ellipsoidal, from colourless to greenish yellow. The oocyst wall is composed of a single layer (about 1 µm), without micropyle. The oocyst residuum and polar granule are absent. The oocyst measurements are given in Table 5.

The sporocysts are ovoid, 5.5 - 7.5 by 3.75 - 5 µm, without Stieda body. The sporocyst residuum is composed of some loose granules.

Localities: The regions of Damascus and Latakia.

Prevalence: *I. anatolicum* was found in 26 of 29 examined animals (20.3%).

Discussion: *I. anatolicum* was first found and described by Sayin et al. (1977) in 1 of the 96 examined specimen of *S. (M.) leucodon* from the region of Ankara (Turkey). According to the original description, the oocysts are spherical, their dimensions vary from 9-11 x 8-9 µm (M = 9.2 x 8.9). It is evident from our data that there are no remarkable differences between our observations and those of Sayin et al. (1977) on *S. (M.) leucodon*. It is interesting to note also that *I. anatolicum* was not established later by Sayin (1980) in the examined 71 specimens of S. *(M.) ehrenbergi* from Southwest Turkey.

As seen from our data, the prevalence of *I. anatolicum* in *S. (M.) ehrenbergi* is higher than in the original host *S. (M.) leucodon*. The similar near morphometric characteristics of the oocysts from the two examined host is a valid reason to accept that *I. anatolicum* is a common protozoan parasite of the herbivorous mole-rats *S. (M.) leucodon* and *S. (M.) ehrenbergi*.

REFERENCES

Darwish A.I., Golemansky V. (1991) Coccidian parasites (*Coccidia: Eimeriidae*) of domestic rabbits (*Oryctolagus cuniculus domesticus* L.) in Syria. Acta Protozool. 31: 209-215.

Sayin F. (1980) Eimeriidae of the herbivorous mole-rat, *Spalax ehrenbergi Nehring*. J. Protozool. 27: 364-367.

Sayin F., Dincer S., Meric I. (1978). Coccidia (Protozoa: Eimeriidae) of the herbivorous mole-rat, *Spalax leucodon* Nordmann. J. Protozool. 24: 210-212.

Veissov A.M. (1975) Three new coccidian from the genus *Eimeria* in the herbivorous mole-rat *Spalax leucodon* Nordmann (1840). Bull. AN Aserb. SSR, Ser. Biol. 4: 82-84 (in Russian).

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ACTA
PROTOZOOLOGICA

Ceratomyxa etroplusi sp.n. (Protozoa: Myxozoa) from the Gall-bladder of the Brackishwater Fish, *Etroplus maculatus* (Bloch)

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Summary. *Ceratomyxa etroplusi* sp. n. producing spores of size 12.38 - 18 x 4.5 - 6.0 µm with asymmetrical shell valves, faint sutural line and sporoplasm filling the entire extracapsular space, recovered from the gall-bladder of *Etroplus maculatus* (Bloch), inhabiting rivulets in Iringal and Ramanattukara of the Calicut district, Kerala, India has been described in detail. This is the first report of a myxosporean from *E.maculatus*.

Key words: *Ceratomyxa etroplusi* sp. n., Myxozoa, parasite of fish *Etroplus maculatus*, India.

INTRODUCTION

Thelohan in 1892 established *Ceratomyxa* with *C. arcuata* as its type. Since then more than 120 species have been added to the genus. Five species of *Ceratomyxa* only have been reported from the Indian fishes (Chakravarty, 1939, 1943; Choudhury and Nandi, 1973; Sarkar, 1986).

While examining the brackishwater fish, *Etroplus maculatus* (Bloch) for their parasites, we came across a myxosporean in their gall-bladder. We ascribe it to *Ceratomyxa*. Detailed observations convince us that the present form is a new species and is reported here as *Ceratomyxa etroplusi* sp. n., after its host.

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MATERIALS AND METHODS

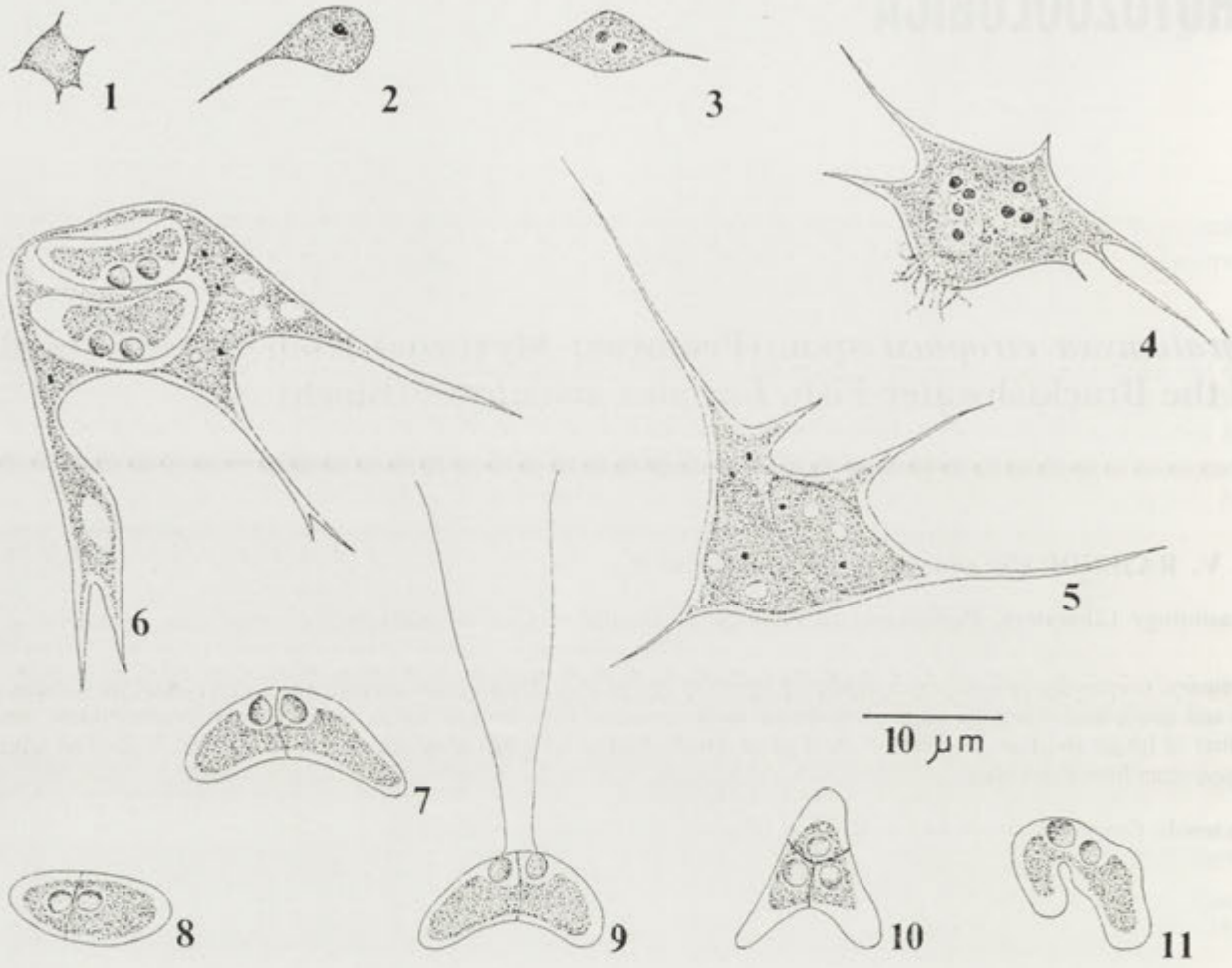
Etroplus maculatus, collected from rivulets in Iringal and Ramanttukara of the Calicut district were brought alive to the laboratory and immediately examined for myxosporeans. Fresh spores and early developmental stages were recovered from the gall-bladder and studied under a phase-contrast microscope. Extrusion of polar filament was achieved with 2% KOH solution. Permanent preparations were obtained by fixing air-dried smears of gall-bladder content on slides in methanol and staining them with Giemsa or fixing them in Schaudinn's fluid and staining with Heidenhain's hematoxylin using eosin as a counterstain. Drawings were made on fresh/stained materials with the aid of a Camera lucida. Measurements are in micrometers. Mean values are given in parentheses.

RESULTS

Ceratomyxa etroplusi sp.n.

Host: *Etroplus maculatus* (Bloch).

Site of infection: Gall-bladder.



Figs 1-11. *Ceratomyxa etroplusi* sp. n. from *Etroplus maculatus* (Bloch). 1 - Smallest trophozoite, 2-3 - Uninucleate and binucleate trophozoites, 4-6 - Trophozoites with developing spores, 7 - Spore - front view, 8 - Spore - capsular view, 9 - Spore with extruded polar filaments, 10-11 - Aberrant spores

Prevalence: Ninety-seven out of 235 examined.

Pathogenicity: Not apparent.

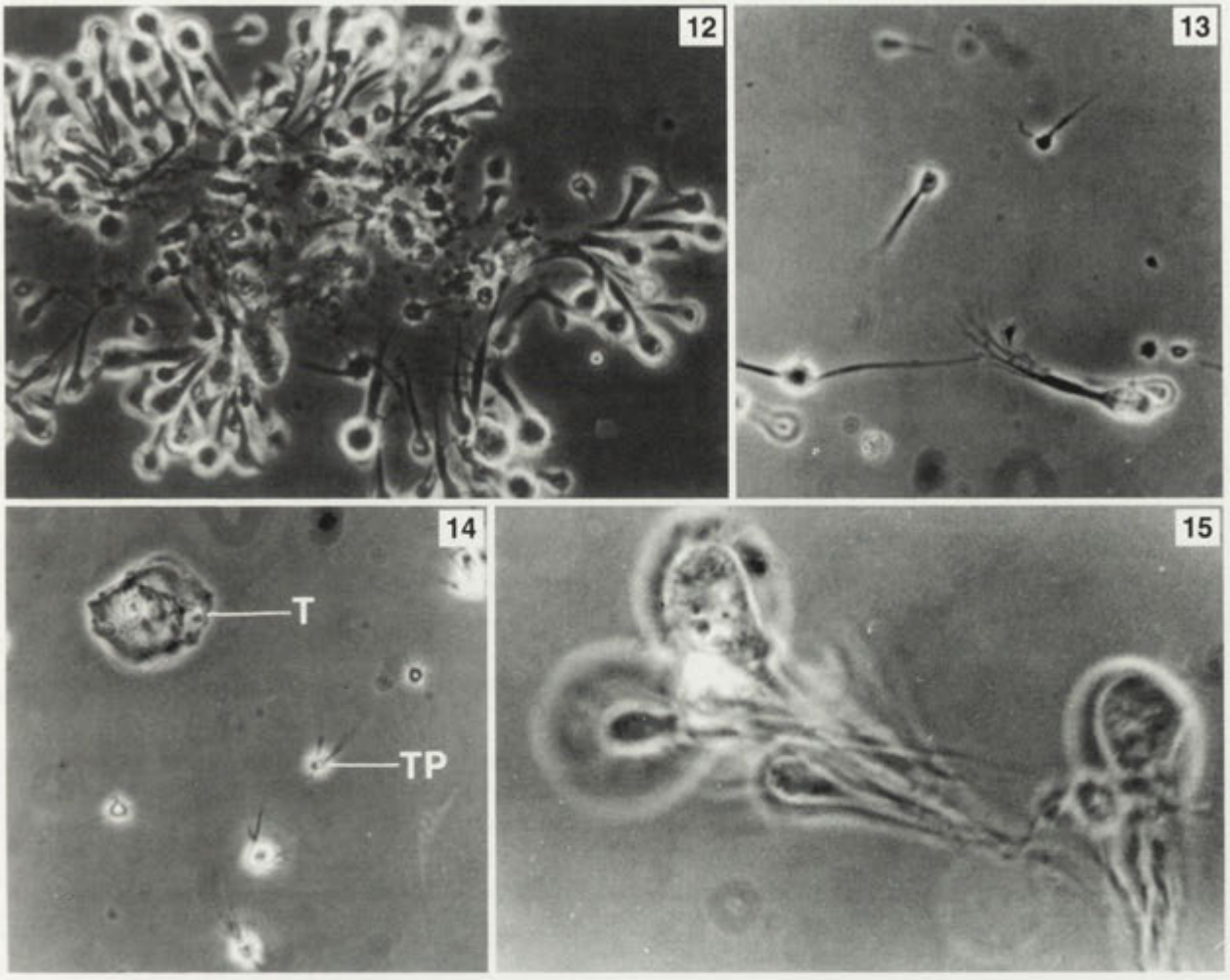
Locality: Rivulets at Iringal and Ramanattukara of the Calicut district of Kerala, India.

Date of collection: February 1988 to July '89.

Type slide: On slide No. M/C-1 kept in the parasite collections, Parasitology Laboratory, Dept. of Zoology, University of Calicut, Kerala (India).

Trophic stages (Figs 1-15): Trophozoites coelozoic, motile or freely floating in bile; variable in shape and size; size range from 3.0 - 53.25 x 3.0 - 18.0 (15.52 x 7.29); with one to several pseudopodia; pseudopodia variable in form, slender and tapering, or filiform. Smallest observed trophozoite round or irregular in outline; cytoplasm finely granular (Fig. 1). Uninucleate trophozoite with single, pointed pseudopodium measures 6.0 x 4.5 (Fig. 2). Binucleate trophozoite possesses two oppositely directed pseudopodia, measures 7.5 x 4.5 (Fig. 3). Trophozoites with developing sporoblasts are larger in size; cytoplasm in them demarcated into hyaline ectoplasm and granular endoplasm; pseudopodia long, drawn out (Fig. 4). Sporulating trophozoites disporous; stage with developing polar capsules measures 15.6 x 10.88 (Fig. 5); those with two well-formed spores measure 31.5 x 18.0 (Fig. 6).

Spore: In sutural view the anterior margin of spores arched, posterior margin moderately to deeply concave



Figs 12-15. Development stages of *Ceratomyxa etroplusi* sp. n. photographed with phase-contrast optics. 12 - Aggregation of trophozoites, 13 - Disporous trophozoite with filamentous pseudopodia, 14 - Trophozoite, pseudopodia withdrawn (T); Young trophozoite with pseudopodia (TP), 15 - Trophozoites, enlarged

(Fig. 7); spores straight in capsular view (Fig. 8); measure, 12.38 - 18 x 4.5 - 6.0 (15.41 x 4.97). Shell valves thin, unequal in most spores; one valve broader and blunter than the other. Suture faintly marked. Polar capsules 2, round to pyriform, opaque, one on each side of the suture; capsules measure, 1.5 - 2.63 x 1.88 - 3.0 (1.95 x 2.66). Extruded polar filaments very thin (Fig. 9). Sporoplasm fills the entire extracapsular space; finely granular with refractile bodies. Sporoplasmic nuclei not visible.

Triad forms (Fig. 10) with 3 valves and 3 polar capsules, and aberrant spores occasionally observed (Fig. 11).

DISCUSSION

The present myxosporean from *Etroplus maculatus* is closely comparable to *Ceratomyxa asymmetrica* Moser et Noble, 1976 from *Coryphaenoides cinereus* of Alaska, *C. castigata* Meglitsch, 1960 and *C. gibba* Meglitsch, 1960 from *Congiopodus leucopaecilis* of New Zealand. All these species infect the gall-bladder of piscine hosts.

The present form is, however, different from *C. asymmetrica* in having smaller spores, a faint sutural line and in the distribution of sporoplasm in the entire extracapsular space; it differs from *C. castigata* in having spores which are more crescentic, with concave posterior margin, smaller sutural diameter and smaller, equal, round to pyriform polar capsules. It is distinctly different from *C. gibba* in having smaller spores and equal polar capsules. Further, the trophic stages of the present form show significant differences from those of the above three species. Moreover, the present species is from a different host, *Etroplus maculatus*. In view of the reasons discussed above the present myxosporean is

considered to be a new species and named *Ceratomyxa etroplusi* sp. n. after its host. This forms the first record of a myxosporean from *E. maculatus*, an important brackishwater fish in India.

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REFERENCES

Chakravarty M. M. (1939) Studies on myxosporidia from the fishes of Bengal, with a note on the myxosporidian infection in aquaria fishes. Arch. Protistenkd. 92: 169-178.

Chakravarty M. M. (1943) Studies on myxosporidia from the common food fishes of Bengal. Proc. Indian Acad. Sci. 18: 21-35.

Choudhury A. and Nandi N. C. (1973) Studies on myxosporidian parasites (Protozoa) from an esturine Gobid fish of West Bengal. Proc. Zool. Soc. (Calcutta) 26: 45-55.

Meglitsch P. A. (1960) Some coelozoic myxosporidia from New Zealand fishes I- General, and Family Ceratomyxidae. Trans. Roy. Soc. of New Zealand. 88: 265-356.

Moser M. and Noble E. R. (1976) The genus *Ceratomyxa* (Protozoa) Myxosporidia) in macrourid fishes. Can. J. Zool. 54: 1535-1537.

Sarkar N. K. (1986) *Ceratomyxa tartoori* sp. n. (Myxozoa) from the gall bladder of a marine teleost (Clupeidae) of West Bengal, India. Acta Protozool. 25: 351-354.

Thelohan P. (1892) Observation sur les myxosporidies et essai de classification de ces organismes. Bull. Soc. Philom. 4: 165-178.

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ACTA
PROTOZOOLOGICA

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Experimental Application of *Nosema* sp. on *Lophocateres pusillus* (K.) (Coleoptera: Curculionidae)

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Summary: Various dosages of the microsporidian parasite, *Nosema* sp. were fed to the 3rd and 4th instar larvae of a major stored grain pest, *Lophocateres pusillus* (K.) (Coleoptera : Curculionidae). A spore dose of 10^5 resulted in 80.76% infection, reduction in larval and adult longevity and a mean spore concentration of 2.42×10^8/dead larva and 2.1×10^8/dead adult. Significant mortality (84.3% larvae and 65.6% adults) occur at the dose of 10^6 spores; this dose produced a mean of 3.15×10^8 spores in dead larvae and 2.5×10^8 spores in dead adults.

Key words: *Lophocateres pusillus*, mortality, *Nosema* sp.

INTRODUCTION

The susceptibility of insects to microsporidian pathogens is well known and has been reviewed by Brooks (1973). In most instances, infected larvae fail to mature, or even if they do, develop into weak adults which are short lived and exhibit reduced fecundity. These detrimental effects generally result from the direct infection of the host insect by the microsporidian pathogen but may also occur when large numbers of ingested spores accumulate in the insect's midgut epithelium, fat bodies, haemocytes and adipose tissues.

Microsporidia of the genus *Nosema* are the most common and widely distributed pathogens infecting natural populations of various field as well as stored grain pests. Because of their wide distribution, common occurrence and apparent pathogenicity, the microsporidia of the genus *Nosema* are promising candidates for the biological control of insect pests (Andreadis 1980, 1984, 1986, Lewis and Lynch 1978, Wilson 1983).

Stored products provide a unique habitat, and it presents a prime opportunity to use protozoans as a preinfestation prophylactic by distributing infective qualities of the pathogens. However, most of the work have been done on field pests. The present paper describes the experimental application of microsporidian, *Nosema* sp. on *Lophocateres pusillus*, a serious stored grain pest.

MATERIALS AND METHODS

Maintenance of insects

Lophocateres pusillus (K.) used in this study were disease free insects reared in the Protozoology Laboratory. Larvae were reared in 100.0 ml glass containers containing sterilized food (autoclave rice dust) until 3rd or 4th instar.

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Collection of spores of Nosema sp.

Spores were obtained from infected *Palorus ratzeburgi* kept in the laboratory. The insects were homogenized in 0.005 M solution of ascorbic acid in 0.135 M NaCl. The homogenate was then washed in a progressively diluted solution of ascorbic acid until the material was transferred to distilled water (Vávra and Maddox 1976).

The resulting homogenate was filtered through two layers of cheesecloth and purified by differential centrifugation. Spores were stored in distilled water at 4°C for 48 h. Spore suspensions were counted using a haemocytometer as described by Cantwell (1970).

Artificial infection

For infecting the larvae with the spores of *Nosema* sp., the measured amount of food material was mixed with 1 ml of spore suspension (1 ml of spore suspension contains 3 x 10^5, 3x10^6 and 3x10^7 spores) and placed in, a Petri dish containing 20 larvae. After allowing to feed for some time the larvae were transferred to fresh vials containing sterilized food. The dose/larva was calculated by the consumption of food materials by the larva. In this way, 462 larvae were fed with *Nosema* sp. spores in three different doses. It was calculated that in three different trials with different doses each larva consumed 10^4, 10^5 and 10^6 spores with food material. Control larvae were treated in the same manner except that they were given glucose water and were maintained simultaneously. Incidence of infection of *Nosema* sp. in the unnatural host was noted up to 120 h post application.

Histopathological study

For histological studies whole larvae were fixed in Carnoy's fluid, washed in 100% ethanol, transferred three times through butyl alcohol and embedded in paraffin. 5-6 µm thick sections were cut and stained in Heidenhain's hematoxylin. Some larvae were fixed in Bouin-Duboscq-Brasil solution overnight, washed in water, dehydrated in graded alcohol, washed in butyl alcohol and embedded in paraffin. 6-8 µm thick sections were cut and stained in modified polychromatic staining method of Vetterling and Thompson (1972).

Study of mortality rate

320 larvae and 320 adults, both disease free, were maintained for the study of mortality rate. Both larvae and adults were grouped into 8 batches of 40 each. Out of the 8 batches of larvae 4 were fed at the dose of 10^5 spores and the rest at 10^6 spores/larva. Adults were maintained similarly. The mortality rate was noted up to 120 h post application. Control batches were fed with distilled water and maintained simultaneously.

Determination of infection intensity

Each insect used for spore count was weighed and homogenized with a tissue grinder. The homogenate was diluted as necessary and spores were counted by the method of Cantwell (1970). Two counts were made for each homogenate and averaged to arrive at the number of individual parasites in the infected host.

RESULTS

Stages of the *Nosema* sp.

Schizont: Small 3-4 µm or large 7-9 µm. All contains diplokaryotic nuclei.

Sporont: Elongated, 5-7 µm.

Spore: Ellipsoidal, measuring 5.1 µm to 8.5 µm ($\bar{x}$ = 7.0 µm) x 4.0 µm to 5.9 µm $\bar{x}$ = 4 mm) in fresh condition.

Experimental infection

After feeding the spores of *Nosema* sp. to the 3rd and 4th instar larvae of *L. pusillus*, examination was commenced at 48 h and continued up to 120 h post application at regular intervals.

At 72 h schizonts with 2-4 nuclei measuring 5-8 µm were found in the gut epithelial cells. After 84 h the polar filament was extruded in most of the spores. At 120 h of infective feed the fat bodies of the hosts were heavily infected by the parasites and the connections between the adjoining cells were lost. Epithelial tissues are greatly hypertrophied. The cytoplasm of the cells of the epithelia become vacuolated and contains sporonts. The epithelial cells were greatly increased due to the presence of large number of spores. Smears prepared at 96 h and 108 h post application showed sporogonial plasmodia after staining with Giemsa's solution.

Histological sections showed that in the gut lumen also are present some spores. This may be due to the rupture of infected cells and to the abnormal regeneration process in the infected midgut epithelium (Lipa et al. 1983). The infected fat cells lost their shape. In extreme parasitemia fat body is diminished which ultimately affects the physiological function of the host.

Incidence of infection

It was observed that the incidence of infection increased with the increase of spore dosage (Table 1). A significant difference in the incidence of infection occurred at different dosages and at different hours. At the dose of 10^6 about 86.95% larvae became infected at 120 h post application. Among the infected individuals 55.0% had heavy infection, 35.0% had medium and 10.0% had trace infection. In general, based on the data of Table 1, the largest spore dose (10^6) as compared to the controls resulted in significant infection in the reared insects.

Table 1

Infection rate in *L. pusillus* experimentally infected with *Nosema* sp. (Figures in parenthesis indicate percentages of infection)

Hours post application	48		60		72		84		96		108		120	
spores / larva	No examined	No infected	No examined	No infected	No examined	No infected	No examined	No infected	No examined	No infected	No examined	No infected	No examined	No infected
10^4	22	1 (4.54)	23	2 (8.7)	20	4 (20.0)	30	8 (26.6)	27	8 (29.62)	24	7 (29.16)	21	8 (38.06)
10^5	23	2 (8.69)	20	3 (15.0)	19	6 (31.57)	22	8 (36.3)	23	12 (52.17)	20	11 (55.00)	26	21 (80.76)
10^6	20	5 (25.0)	19	7 (36.8)	19	8 (42.1)	23	11 (47.8)	20	10 (50.00)	18	11 (61.11)	23	20 (86.95)
Control	15	0	15	0	20	0	15	0	13	0	17	1 (5.88)	20	1 (5.00)

Rate of mortality of larvae and adults

After 120 h, at both the doses (10^5 spores/individual, and 10^6 spores/individual) significant mortality occurred in both larvae and adults. In the case of larvae altogether 78.7% died at the dose of 10^5 and 84.3% at the dose of 10^6 (Fig.1). In the case of adults, dose of 10^5 and 10^6 resulted in altogether 57.4% and 65.6% mortality, respectively (Fig. 2). During the experiment mortality among control larvae and adults was insignificant.

Microscopic examination of dead larvae showed hypertrophied epithelial cells and fragmented fat bodies. These changes were probably the cause of larval death.

Of the survivors majority showed symptoms of infection i.e., larvae became brownish and lethargic; adults showed less appetite, decreased size and weight and a tendency for aggregation.

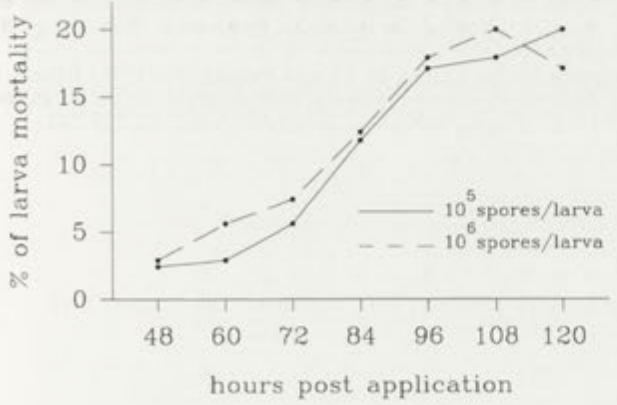

Fig 1. Percent of mortality of *L. pusillus* (K.)(larvae) after experimentally infected with *Noesema* sp. at the dose of 10^5 and 10^6 spores/individual

Intensity of infection of *Nosema* sp. in dead larvae and adults of *L. pusillus*

The mean number of spores in dead larvae is 2.42×10^8/mg of body weight at the dose of 10^5 and 3.15×10^8 spores/mg body weight at the dose 10^6, whereas in the dead adult 2.1×10^8 spores/mg body weight at 10^5 and 2.5×10^8 spores/mg body weight at 10^6 (Table 2). It was observed that weights of larvae and adults decreased due to infection.

DISCUSSION

Lethal effects of microsporidian infection include reduced pupal weight, fecundity and adult longevity. Gaugler and Brooks (1975) fed 3 days old larvae of *Heliothis zea* on diet treated with 1.3 x 10^6 spores of *Nosema heliothidis*. Longevity of adults from these larvae was shortened by about 4 days. Longevity of male and female moths of *Ostrinia nubilalis* naturally infected with *Nosema pyrausta* which exceeded a concentration of 10^6 spores, lived an average of 2.0 and 4.2, fewer

Table 2

Intensity of *Nosema* sp. infection in larvae and adults of *L. pusillus* following ingestion of spores by 3rd and 4th instar larvae (Figures in brackets indicate number of individual used in the calculations)

Doses of spore / larva	Mean dry wt (mg)		Mean No x $10^8 \pm$ S.E Spores / mg tissue	
	Larvae	Adult	Larvae	Adult
10^5	8.21 (54)	11.40 (19)	2.42 ± 0.4	2.1 ± 0.2
10^6	10.05 (70)	20.35 (48)	3.15 ± 0.3	2.5 ± 0.3

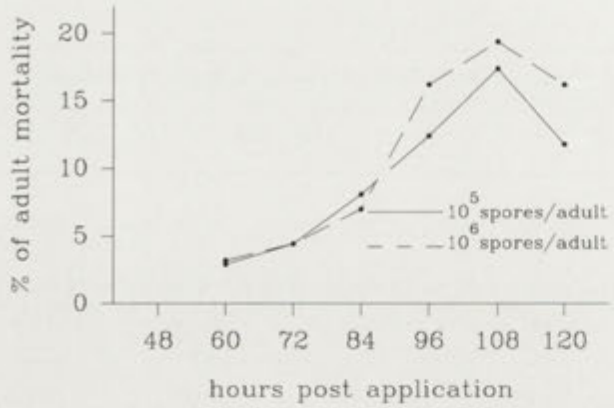

Fig 2. Percent of mortality of *L. pusillus* (K.)(adults) after experimentally infected with *Noesema* sp. at the dose of 10^5 and 10^6 spores/individual

days, respectively, than did moths having no infection (Windles et al. 1976).

In general, the mortality of insects is related to the size of the initial dose of parasite received. Thompson (1958) infected spruce budworm with *N. fumiferanae* by spraying spore suspension on foliage in the laboratory. A dosage of $3x10^7$ spores/ml resulted in 40% larval mortality over a period of 25 days. Ghosh (1990) in his experiment on stored pest, *Oryzaephilus mercator* showed that application of $4x10^6$ spores/ml causes 70% larval and 58.5% adult mortality at the end of seven days.

The number of spores (intensity of infection) that an host can harbour and still function normally is important in determining the role of a microsporidian as parasite in nature. A dose of 10^5 *Nosema carpocapsae* spores fed to 4th instar larvae of *Cydia pomonella* resulted in $8x10^6$ spores /adult, whereas feeding 10^8 spores gave $2.9x10^7$ spores/adult (Malone and Wigley 1981). Although the infection did not cause mortality, it did reduce the fecundity and fertility of infected moths. Windles et al. (1976) reported an average intensity of natural infection of *N. pyrausta* in *O. nubilalis* males and females as 25.4×10^6 and 38.9×10^6 spores/moth, respectively, these levels being detrimental to the host. In the case of the stored pest, *Lophocaleres pusillus*, 3.15×10^8 spores/larva and 2.5×10^8 spores/adult is highly effective and cause mortality of 84% larvae and 62% adult.

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REFERENCES

Andreadis T. G. (1980) *Nosema pyrausta* infection in *Macrocentrus grandii*, a braconid parasite of the European corn borer, *Ostrinia nubilalis* J. Invertebr. Pathol. 35: 229-233.

Andreadis T. G. (1984) Epizootiology of *Nosema pyrausta* in field population of the European corn borer (*Lepidoptera*: Pyralidae). Env. Entomol. 13: 882-887.

Andreadis T. G. (1986) Dissemination of *Nosema pyrausta* in feral populations of the European corn borer, *Ostrinia nubilalis*. J. Invertebr. Pathol. 48: 335-343.

Brooks W. M. (1973) Protozoa: Host parasite-pathogen interrelationships. Misc. Pub. Entomol. Soc. Am. 9: 105-111.

Cantwell G. E. (1970) Standard methods for counting *Nosema* spores. American Bee J. 95: 52-53.

Gaugler R. R. and Brooks W. M. (1975) Sublethal effects of infection of *Nosema heliothidis* in the corn earworm, *Heliothis zea*. J. Invertebr. Pathol. 26: 57-63.

Ghosh S. (1990) Experimental application of *Microsporidium sitophili* Ghose, on major stored grain pest, *Oryzaephilus mercator* (F.) infesting nuts, *Anacardium occidentalis* and *Arachis hypogea*. Acta Protozool. 29: 257-264.

Lewis L. C. and Lynch R. E. (1978) Foliar application of *Nosema pyrausta* for suppression of European corn borer. Entomophaga 23: 83-88.

Lipa J. J., Amargier A. and Vago C. (1983) Histopathology of a microsporidian infection caused by *Pleistophora legeri* (Paillot) in *Lobesia botrana* Den. et. Schiff. (Lepidoptera, Tortricidae). Acta Protozool. 22: 261-266.

Malone L. A. and Wigley P. J. (1981) Quantitative studies on the pathogenicity of *Nosema carpocapsae*, a microsporidian pathogen of the codling moth, *Cydia pomonella*, in New Zealand. J. Invertebr. Pathol. 38: 330-334.

Thompson H. M. (1958) The effect of a microsporidian parasite on the development, reproduction and mortality of the spruce budworm, *Choristoneura fumiferana* (Clem.). Canad. J. Zool. 36: 499-511.

Vávra J. and Maddox J. V. (1976) Methods in Microsporodiology. In: Comparative Pathobiology. Eds. L.A. Bulla & T. C. Cheng, pp. 282-319. Plenum Press, New York, London.

Vetterling J. M. and Thompson D. E. (1972) A polychromatic stain for the use in parasitology. Stain Technol. 47: 164-165.

Wilson G. G. (1983) A dosing technique and the effects of sublethal doses of *Nosema fumiferanae* (Microsporida) on its host the spruce budworm, *Choristoneura fumiferana*. Parasitology 87: 371-376.

Windles M. B., Chiang H. C. and Furgala, B. (1976) Effects of *Nosema pyrausta* on pupa and adult stages of the European corn borer, *Ostrinia nubilalis*. J. Invertebr. Pathol. 27: 239-242.

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