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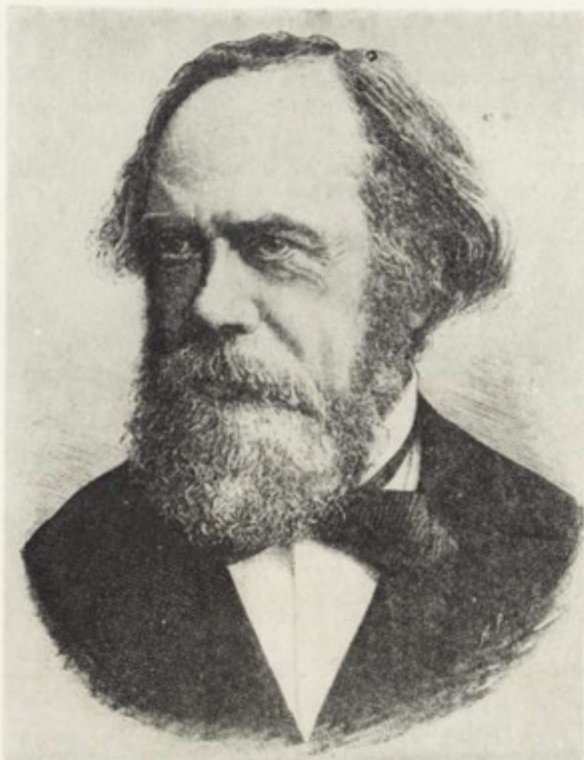

Leon CIENKOWSKI

Born on 1 October 1822 in Warsaw
Died on 8 October 1887 in Leipzig

One hundred years ago Leon Cienkowski one of the leading protistologists and microbiologists in the second half of the XIX century passed away as a victim of cancer.

Leon Cienkowski at the age of seventeen lived in Warsaw where he graduated from the high school. In 1839 he was passing the examination as a fellowship holder of the Polish Kingdom and attended the St. Petersburg University, where he received the candidate degree in 1844 and master degree in 1846. In 1847–1849 Leon Cienkowski was nominated a member of the Russian scientific expedition to Egypt and Sudan.

He was a professor in Botany at the University of St. Petersburg, from 1855 to 1859, at the New-Russian University of Odessa (1865–1871) at the University of Kharkov from 1827 to 1887. Leon Cienkowski carried out research not only in Russia and in Ukraine, but also in many scientific centers of Western Europe in the years 1859–1865 and later during visits to Germany and France. He published scientific papers in German and Russian and popular scientific articles in Polish.

Leon Cienkowski was widely recognized and respected for his work on all major groups of Protista: rhizopods, chlorobionts, labyrinthomorphs, actinopods, dinoflagellates and ciliates. He was one of the pioneer of comparative cytology. On the basis of microscopic research he rejected Stein's "Acineten-theory" (1855) and successfully criticized Ehrenberg's ideas on "Infusionthierchen as vollkommene Organismen". In 1865 (ten years before E. Haeckel), Cienkowski postulated that no border exists between Animals and Planta. Protozoa and Algae are artificial groups, because both represent the same kind and degree of cellular organization.

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In sixties (1862, 1863) Cienkowski studied myxomycetes and gave the description of reproduction and life cycle, of these organisms. He introduced the term "plasmodium" and he was the first one, who established the acellular stage of myxomycetes as one of the most favorable material for the analysis of primitive motile systems. Till recently he was known especially for his classical papers on genus *Labyrinthula* 1867, *Clathrulina* 1867 and *Noctiluca miliaris* 1871, 1873.

Leszek KUŹNICKI and Stanisław DRYL

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Morphologie von *Holosticha xanthichroma* sp. n. und die Variabilität der Infraciliatur in der Gattung *Holosticha* (*Ciliophora*, *Hypotrichida*)

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Received on 14 August 1986

Synopsis. *Holosticha xanthichroma* sp. n. wurde in einem alpinen Kleingewässer gefunden und unterscheidet sich von den anderen Arten dieser Gattung durch das diffus gelb gefärbte Cytoplasma. Diese Färbung läßt sich lichtmikroskopisch nicht auf Pigmentgranula zurückführen. Die Variabilität der meisten Merkmale liegt beträchtlich über dem Durchschnitt von 10 anderen *Holosticha*-Populationen; Hinweis auf Zwillingsarten.

Wichtige Art-Kriterien der Gattung *Holosticha* sind nach den bisher vorliegenden biometrischen Analysen der Infraciliatur die Anzahl der Dorsalkineten, Buccalcirren, Parabuccalcirren, adoralen Membranellen, Midventralcirren und Marginalcirren sowie die Länge der adoralen Membranellenzone und Midventralreihen und offene oder überkreuzte Marginalreihen. Die Anzahl der Transversalcirren variiert sehr stark.

Als Wrześniowski (1877) für oxytrichide Taxa mit „continuirlichen Bauchwimperreihen" den neuen Namen *Holosticha* einführte, bezog er ihn auf insgesamt 7 Species. Mittlerweile liegen etwa 49 Artbeschreibungen vor, die meisten stammen von Kahl (1928, 1932). Als Abgrenzungskriterien dienten früher die Gestalt, der Lebensraum, die Anzahl der Makronucleus-Teile und der Transversalcirren, später auch die Infraciliatur (Borror 1972, Hemberger 1982) und subpelliculäre Granula (Foissner und Didier 1981, Foissner 1982, Borror und Wicklow 1983). Das Ausmaß der Variabilität wurde von Hemberger (1982) und Borror und Wicklow (1983) — ohne statistische Kennzahlen — angegeben und erst bei einigen Arten detailliert untersucht (Foissner 1982). Daher ist die Identifikation meist sehr schwierig. Dies dokumentiert die beinahe willkürliche Anerkennung und Ablehnung von Arten (Kahl 1932; Borror 1972, Hemberger 1982, Borror und Wicklow 1983).

Im vorliegenden Beitrag werden neben der Beschreibung einer neuen Species die bekannten biometrischen Daten von *Holosticha*-Arten verglichen und bewertet.

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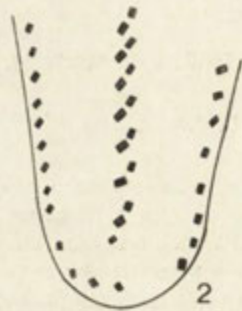

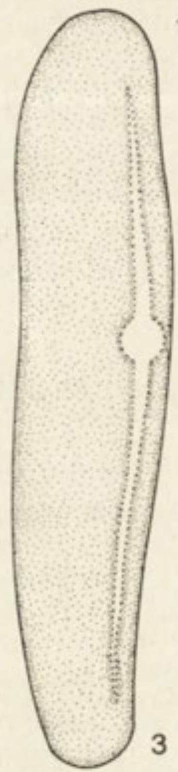

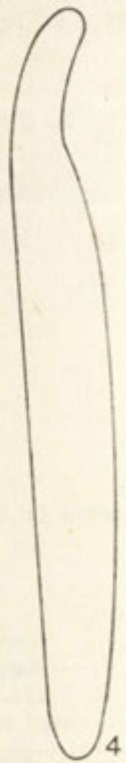

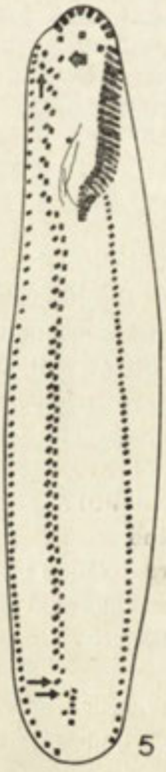

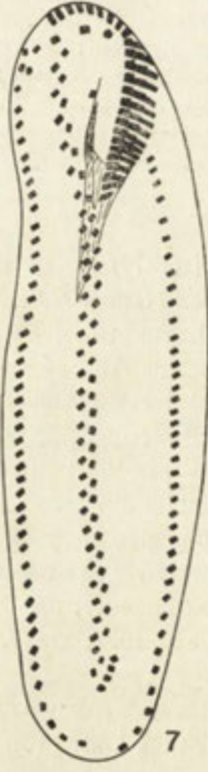

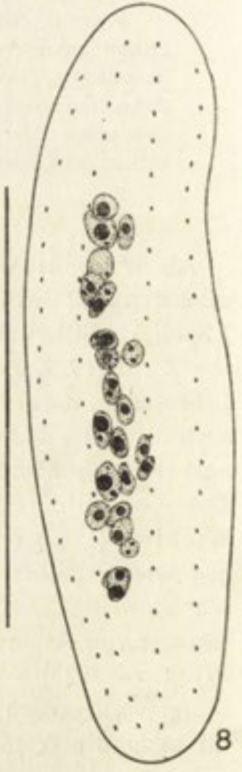

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Material und Methoden

Holosticha xanthichroma fanden wir im Uferbereich eines alpinen Weidetümpels auf der Schloßalm bei Bad Hofgastein. Die Körperform ist nach Präparaten gezeichnet, die nicht mit einem Deckglas bedeckt waren. Später wurden die Tiere durch leichten Deckglasdruck festgelegt und mit dem Ölimmersionsobjektiv im Hellfeld untersucht. Die Imprägnation der Infraciliatur erfolgte mit Protargolsilber nach der Methode von Foissner (1982). Die entsprechenden Zeichnungen sind mit einem Zeichenapparat angefertigt und nur sehr wenig schematisiert. Die statistischen Kennzahlen richten sich nach Foissner (1982).

Terminologie nach Kahl (1932). Neuere Vorschläge von Borror (1972, 1979) und Borror und Wicklow (1983) erscheinen uns eher verwirrend (z.B. nennt Borror den l. Frontalcirrus 1972 Buccalcirrus, später dann Paroralcirrus). Wir anerkennen jedoch, daß eine differenziertere Bezeichnung der Cirren erforderlich ist und nennen daher die Cirren zwischen den Buccalcirren und Midventralreihen (in Anlehnung an Borror und Wicklow 1983) Parabuccalcirren. Jene Cirren zwischen Transversalcirren und zickzack-förmig angeordneten Midventralreihen bezeichnen wir als Ventralcirren.

Ergebnisse und Diskussion

Beschreibung von *Holosticha xanthichroma* sp. n. (Abb. 1. *1–8*, Tabelle 1)

Diagnose: *In vivo* etwa 110–220×26–45 μm große, leicht kontraktile, linealische *Holosticha*, deren Plasma gelb gefärbt ist. Midventralreihen unverkürzt. Vier Dorsalkineten. Durchschnittlich 28 ellipsoide Makronucleus-Teile.

Locus typicus: Selten am Ufer eines perennen, leicht verschmutzten Weidetümpels auf der Schloßalm bei Bad Hofgastein (1950 m ü. d. M.), Bundesland Salzburg, Austria.

Aufbewahrungsort des Typenmaterials: Ein Holo- und ein Paratypuspräparat sind in der Sammlung der mikroskopischen Präparate des Oberösterreichischen Landesmuseums in Linz (Österreich) deponiert. Als Holotypus ist ein Individuum mit mehreren Transversalcirren festgelegt.

Morphologie: Meist leicht S-förmig gebogen und hinten quer abgestutzt, Vorderende abgerundet (Abb. 1 *1*, *2*). Ungefähr 1.5–2:1 abgeflacht (Abb. 1 *4*). Makronucleus-Teile *in vivo* 4–13×3–4 μm groß, längs der Medianen liegend, Übergänge zwischen vielen kleinen und wenigen großen Chromatinkörpern. Meist 3 schwach argyrophile Mikronuclei, *in vivo* 4–6 μm durchmessend (Abb. 1 *6*, *8*). Kontraktile Vakuole etwas vor der Körpermitte am linken Körperrand, während der Diastole 2 Zuführungskanäle, die fast die Körperenden erreichen (Abb. 1 *3*).

Abb. 1 *1–8*. *Holosticha xanthichroma* nach Lebendbeobachtungen (*1*, *3*, *4*) und Protargolimprägnation (*2*, *5–8*). *1* — Ventralansicht. *2* — Posteriore Körperregion ohne Transversalcirren. *3* — Formvariante in Dorsalansicht. *4* — Seitenansicht. *5–8* — Infraciliatur der Ventral- und Dorsalseite. Die vorderen Pfeile weisen auf die Frontoterminalcirren und den Parabuccalcirrus, die hinteren auf die Ventralcirren. Maßstriche: 60 μm

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Tabelle 1
Biometrische Charakteristik von *Holosticha xanthichroma*

Merkmal	$\bar{x}$	M	SD	SE	V	Min	Max	*n*
Länge in μm	126.2	122.0	24.7	4.9	19.5	90	173	26
Breite in μm	30.9	30.0	4.6	0.9	15.0	25	45	26
Länge eines Ma-Teiles	5.5	5.0	2.5	0.3	45.1	2	10	52
Breite eines Ma-Teiles	2.9	3.0	0.9	0.1	32.1	2	5	52
Länge der AZM in μm	34.5	33.5	6.9	1.4	20.0	22	50	26
Anzahl der Ma-Teile	28.4	28.5	7.7	1.5	27.1	11	42	26
Anzahl der AM	37.0	38.5	6.9	1.4	18.8	21	46	26
Anzahl der RMC	49.4	49.5	12.3	2.4	25.0	26	72	26
Anzahl der LMC	53.5	56.0	12.7	2.5	23.8	32	74	26
Anzahl der RMVC	41.2	40.0	10.4	2.0	25.3	20	62	26
Anzahl der LMVC	41.7	41.0	10.4	2.0	24.9	22	65	26
Anzahl der FC	3.0	3.0	0.0	0.0	0.0	3	3	26
Anzahl der BC	1.0	1.0	0.0	0.0	0.0	1	1	26
Anzahl der PBC	1.0	1.0	0.0	0.0	0.0	1	1	26
Anzahl der FTC	2.0	2.0	0.3	0.1	14.0	1	3	26
Anzahl der TC	4.0	4.0	1.9	0.4	48.2	0	6	26
Anzahl der DK	4.0	4.0	0.0	0.0	0.0	4	4	26
Länge : Breite	4.1	4.4	0.7	0.1	16.5	3	6	26
Länge : Länge der AZM	3.7	3.7	0.5	0.1	13.4	3	5	26

Alle Daten basieren auf protargolimprägnierten Individuen. AM — adorale Membranellen, AZM — adorale Membranellenzone, BC — Buccalcirren, DK — Dorsalkineten, FC — Frontalcirren, FTC — Frontoterminalcirren, LMC — linke Marginalcirren, LMVC — linke Midventralcirren, M — Median, Ma — Makronucleus, Max — Maximum, Min — Minimum, *n* — Anzahl der untersuchten Individuen, PBC — Parabuccalcirren, RMC — rechte Marginalcirren, RMVC — rechte Midventralcirren, SD — Standardabweichung, SE — Standardfehler des Mittelwertes, TC — Transversalcirren, V — Variabilitätskoeffizient, $\bar{x}$ — arithmetisches Mittel.

Pellicula sehr weich und biegsam, entlang der Cirrenreihen kreneliert. Entoplasma auffallend gelb gefärbt (erscheint bei kleiner Vergrößerung bräunlich), manchmal mit mäßig vielen, am Hinterende gehäuften, 2 μm großen, dunklen Einschlüssen. Nahrungsvakuolen mit Diatomeen und undefinierbarem Inhalt. Ständig in Bewegung, schwimmt gleitend oder kriecht wurmartig über das Substrat.

Adorale Membranellenzone etwa 1/4 körperlang, Pharynxfibrillen sehr fein und lang, Buccalfeld klein, nicht auffallend eingesenkt. Parorale und endorale Membran sehr kurz, leicht gebogen (Abb. 1 *5*, *7*). Drei gering verstärkte Frontalcirren, 1 zarter Buccalcirrus, 1 leicht verstärkter Parabuccalcirrus, 2 weit nach rechts gerückte Frontoterminalcirren. Midventralreihen beginnen knapp unter dem 3. Frontalcirrus, ziehen leicht S-förmig bis vor die Gruppe der Ventral- und Transversalcirren. Transversalcirren kurz, überragen den hinteren Körperrand nicht, in sehr steiler Reihe, bei 22 von 79 Individuen (28%) fehlten sie (Abb. 1 *2*). Marginalreihen hinten offen, rechte am posterioren Körperende mehr oder weniger deutlich gebogen (Abb. 1 *5*, *7*). 1—2 der 4 Dorsalkineten anterior oder posterior mehr oder weniger stark verkürzt (Abb. 1 *6*, *8*). Vielleicht 1—3 aus wenigen Cilien aufgebaute Caudalcirren.

Artvergleich: Von allen bisher bekannten Arten dieser Gattung unterscheidet sich *Holosticha xanthichroma* durch das auffallend gelb gefärbte Plasma, wobei

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die eigene Kenntnis vieler anderer hypotricher Ciliaten ein optisches Artefakt ausschließt. Möglicherweise ist diese diffuse Färbung auf feinste Pigmentgranula zurückzuführen, die lichtmikroskopisch nicht auflösbar sind. Hinsichtlich der Infraciliatur ähnelt *H. xanthichroma* sehr *H. grisea* Kahl, 1932, *H. violacea* Kahl, 1928 und einer *Keronopsis monilata* Variante von Kahl (1932; Seite 577, Abb. 1), die wegen der Frontalciliatur vermutlich zur Gattung *Holosticha* gehört. Dies unterstützt die bereits früher dargelegte Auffassung (Foissner und Adam 1983, Borror und Wicklow 1983), daß neuere Bestrebungen, die Artbeschreibung von hypotrichen Ciliaten auf die Darstellung der Infraciliatur und des Kernapparates zu reduzieren, unrichtig sind.

Aus den Variabilitätskoeffizienten von 8 „geläufigen" Merkmalen mehrerer *Holosticha*-Arten wird deutlich, daß *H. xanthichroma* mit Variabilitätskoeffizienten zwischen 15% und 25% beträchtlich über dem Durchschnitt der anderen Species liegt, was auf Zwillingsarten hindeutet (Tabelle 2).

Tabelle 2
Variabilitätskoeffizienten in der Gattung *Holosticha* im Vergleich zu jenen von 34 verschiedener hypotricher Ciliaten-Arten

Species		Länge	Breite	AZML	AM	RMC	LMC	RMVC	LMVC
H. adami (Foissner 1982)		16.1	16.0	11.8	11.8	12.5	12.6	14.0	14.2
H. multistilata (Foissner 1982)		13.1	10.8	11.0	9.2	10.0	11.5	9.8	11.9
H. muscorum (Foissner 1982)		10.2	18.6	10.9	12.6	10.9	22.0	12.5	17.0
H. similis (Foissner und Didier 1981)		13.8	10.3	11.9	12.2	20.0	15.2	11.3	11.3
H. sylvatica (Foissner 1982)		6.9	13.4	7.3	4.4	10.7	9.4	14.3	14.3
H. sigmoidea	P1	11.2	16.0	5.6	13.0	14.2	15.8	8.8	9.8
(Foissner 1982)	P2	7.5	11.7	5.0	5.0	8.8	15.9	13.7	16.6
(Foissner 1984)	P3	13.8	9.8	8.6	4.2	9.2	9.5	10.8	10.8
H. tetracirrata	P1	6.0	17.4	8.1	2.9	5.6	10.9	12.6	8.1
(Foissner 1982)	P2	12.6	13.2	9.7	2.8	8.3	9.3	11.9	14.1
H. xanthichroma		19.5	15.0	20.0	18.7	25.0	24.0	25.3	25.0
$\bar{x}$ 11 *Holosticha*-P		11.9	13.8	10.0	8.8	12.3	14.2	13.2	13.9
$\bar{x}$ 42 hypotriche P (Foissner 1982)		11.5	13.6	9.5	7.3	10.1	11.0	—	—

Alle Daten basieren auf protargolimprägnierten Individuen. AM — adorale Membranellen, AZML — Distanz anteriores Körperende bis zur letzten AM, LMC — linke Marginalcirren, LMVC — linke Midventralcirren, P — Population, RMC — rechte Marginalcirren, RMVC — rechte Midventralcirren, $\bar{x}$ — arithmetisches Mittel.

Variabiliät in der Gattung *Holosticha*

Foissner (1982, 1984) charakterisierte zahlreiche hypotriche Ciliaten biometrisch und betonte, daß der Variabilitätskoeffizient (Standardabweichung durch Mittelwert in Prozent) auf lange Sicht eine ausgezeichnete Möglichkeit bietet, die

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konstanten oder wenig variablen Merkmale einer Art oder einer bestimmten Gruppe herauszufinden. Innerhalb der *Hypotrichida* ergaben die Anzahl der Dorsalkineten, der Buccal-, Frontal- und Marginalcirren sowie die Anzahl der adoralen Membranellen und die Länge der adoralen Membranellenzone eine geringe Variabilität, wodurch diese Merkmale für die Feinsystematik eine große Bedeutung erlangen, zumal sie eine sehr geringe Uniformität aufweisen.

Wie verhält es sich nun mit der Variabilität dieser Merkmale innerhalb der Gattung *Holosticha*? Die Anzahl der Dorsalkineten ist bei den bisher untersuchten Arten und Populationen weitgehend konstant: 3 bei *H. adami*, *H. multistilata* und *H. muscorum*; 4 bei *H. sigmoidea* (3 Populationen), *H. tetracirrata* (3 Populationen; vgl. Foissner 1982, Buitkamp und Wilbert 1984) und *H. xanthichroma*; 5 bei *H. sylvatica* und 6 bei *H. similis* (Foissner 1982, 1984, Foissner und Didier 1981). Die Anzahl der Buccalcirren ist bei den monocirraten Species (z. B. *H. xanthichroma*, *H. sylvatica*) fast konstant (bei *H. similis* fehlt er manchmal, $\bar{x} = 0.6$), bei den polycirraten Arten (z. B. *H. multistilata*) sehr variabel (Foissner 1982). Bemerkenswert ist, daß bei den polycirraten Taxa die Buccalcirren nie einzeln vorkommen oder fehlen. Daher kann mit diesem Merkmal *Holosticha* in 2 Gruppen aufgetrennt werden: Arten mit 0 bis 1 oder 2 bis vielen Buccalcirren. Genauer sollten in Zukunft die Cirren im Frontalabschnitt beachtet werden, da es vielleicht Arten gibt, die außer den üblichen 3 vorderen verstärkten Frontalcirren und einem (Bucalcirrus) keine oder mehrere Parabuccalcirren zwischen den Midventralreihen und Buccalcirren aufweisen. Die Parabuccalcirren sind ihrer morphogenetischen Herkunft nach eindeutig von den Midventralcirren zu unterscheiden (z. B. *H. multistilata* in Hemberger 1982). Zur Zeit werden sie biometrisch zu uneinheitlich behandelt (Foissner 1982, 1984). Eine ähnliche Situation besteht bei den Ventralcirren vor den Transversalcirren. Obwohl sie durch ihre Position innerhalb der Cirrenanlagen bei der Morphogenese differenziert werden können, ist ihre Unterscheidung beim Interphase-Individuum oft nicht eindeutig möglich. Derzeit werden sie meist den Midventralcirren zugezählt. Ein wenig brauchbares Merkmal für *Holosticha*-Arten ist die Anzahl der Transversalcirren, die häufig sehr hohe Variabilitätskoeffizienten haben (z.B. 44% bei *H. tetracirrata* (Foissner 1982) und 48.2% bei *H. xanthichroma*). Die Anzahl der adoralen Membranellen und die Länge der adoralen Membranellenzone sind auch in dieser Gattung wenig variabel und daher für die Charakterisierung der Arten besonders wichtig (Foissner 1982).

Eine weitere Gruppenbildung ist mit der Länge der Midventralreihen möglich: sie reichen entweder bis zu den Ventralcirren oder sind mehr oder weniger stark verkürzt. Die Variabilität dieses Merkmals ist mit etwa 8—15% niedrig genug, um es taxonomisch verwerten zu können. Auch hinsichtlich der Marginalreihen lassen sich zumindest zwei Gruppen unterscheiden: bei einer sind sie hinten offen (z.B. *H. xanthichroma*), bei der anderen überkreuzt (z. B. *H. muscorum* in Foissner 1982). Das Ausmaß der Variabilität der Anzahl der Marginalcirren liegt in der Größenordnung jener der Midventralcirren.

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SUMMARY

Holosticha xanthichroma nov. spec. occurred in a small alpine pool and differs from the other members of the genus by its yellow coloured cytoplasm. This colour is not caused by lightmicroscopically recognizable pigment granules. The variability of most characters of *H. xanthichroma* is considerably higher than the mean of those from 10 other populations of *Holosticha* spp. May be it is a complex of sibling species.

A comparison of the available biometrical data suggests that the following characters of the infraciliature are especially useful for *Holosticha* species: number of dorsal kineties, buccal cirri, parabuccal cirri, midventral cirri, marginal cirri, adoral membranelles; length of adoral zone of membranelles and midventral rows; crossed or open marginal rows.

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Some Ultrastructural Changes Produced by Vaccinia Virus in *Onychodromus acuminatus* (*Ciliata*, *Hypotricha*)

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Synopsis. The hypotrich ciliate *Onychodromus acuminatus* shows in its cytoplasm a proliferation of membranous cisternae after 2 h of culture inoculation by vaccinia virus. The stacks of small vesicles lie near the mitochondria and they are formed by budding from the smooth part of the endoplasmic reticulum. These groups of vesicles disappear when the viruses are eliminated. Despite the fact that vaccinia virus does not leave the digestive vacuoles – at least I have never found virions free in the cytoplasm – some substances produced in the degradation of these viruses could be released into the cytoplasm. The hypertrophy of these structures could be related to the production of some substances to control or diminish the possible cytopathic effects of viruses.

In a preliminary communication we have shown (Jareño et al. 1980) that some large ciliates such as *Onychodromus acuminatus* could be interesting tools in cytological studies of cell–virus interactions at ultrastructural level. Ciliates and virus have never been used in these kind of ultrastructural studies. The works concerning ciliates–virus relationship published until now deal only with physiological interrelations (Brutsaert et al. 1946, Evans and Osterud 1946, Groupé and Pugh 1952, Groupé et al. 1955, Kovacs et al. 1966, Kovacs and Buzc 1967, Kesa and Teras 1981, Pérez and García 1981).

We have also verified that some species of free living ciliates as *Onychodromus acuminatus*, are able to eliminate in 2 days the vaccinia virus added to the ciliate culture. The tritations were made by plaque assay in chick embryo cell monolayers (Pérez et al. 1983).

This paper shows at ultrastructural level some morphological changes produced in the cytoplasm of *Onychodromus acuminatus* by vaccinia virus ingestion.

Materials and Methods

The strain of *Onychodromus acuminatus* and culture method have been described previously (Jareño 1977). *Paramecium bursaria* was isolated from river water, maintained in the laboratory at 21°C in soil medium and fed with *Chlorogonium* sp. This is cultivated axenically in Pringsheim's medium.

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For this cytological study, I have started from a stock of virus originally 1.4×10^6 PFU/ml. Five millilitres of this stock were centrifuged at 30000 rpm. for 1 h and the pellet of cells with virus were introduced to soil medium with 100 cells/ml of ciliates.

During the first 3 days of the experiments the remnants of cells in the virus culture were the only food. Afterwards the ciliates were normally fed with *Chlorogonium* sp.

Three types of experiments have been made:

(1) The culture of protozoa was inoculated with the virus, and samples of ciliates were taken at different intervals of time.

(2) Some individuals were picked up 2 h after culture inoculation, washed thoroughly to eliminate the virus and maintained in a virus free medium.

(3) The culture of protozoa was inoculated during 5 consecutive weeks, once each week. Some ciliates were picked up half an hour before the fifth inoculation and others half an hour after this.

All samples were processed for ultramicroscopical observations as follows: organisms were fixed for 30 min at laboratory temperature in a mixture (1:1) of 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and 2% OsO_4 in distilled water, washed in the same buffer, postfixed in 2% OsO_4 in distilled water for 30 min at room temperature and embedded in Araldite. Ultrathin sections were obtained with a glass knife on LKB ultramicrotome. They were mounted on grids coated with formvar film and stained with uranyl acetate for 10 min and lead citrate for 15 min.

Observations

Although vaccinia virus morphology is commonly known, we show here, for better understanding, its schematic design (Pl. I 1) and appearance in the food vacuoles of *Onychodromus acuminatus* (Pl. I 2). These figures show the three major components of a vaccinia virus: a lipoprotein membrane, the core (c) and the two lateral bodies (LB). This virus belongs to the poxvirus group and replicates within the cytoplasm near or inside sites called "factories". Vaccinia virus has some favourable characteristics for cytological studies at ultrastructural level: it is a large virus (230—300 nm), its ultrastructure is very different from the structures of protozoa cells and it survives for the time of the experiments in the environmental conditions where the protozoa are cultivated.

Macroscopic and microscopic observations of ciliates at different intervals of time from the culture inoculation and subsequent ingestion of the virus, indicate that the ciliates are not affected in either their motility or division rate. The ciliates encyst normally when starved, even in those cultures which were inoculated several times, once a week. The only change observed in the ciliates was at ultrastructural level.

Half an hour after culture inoculation the cells of *O. acuminatus* show large vacuoles with many virions inside. Plate I 3 shows many virions in different phases of degradation in a digestive vacuole. We can also see vacuoles with viruses in different phases of degradation in cells that have remained up to 48 h with the virus; they are not seen after this time. When the individuals have remained with the virus 2 h and then were washed thoroughly in soil medium to separate the virus, no recognizable particles of the virus were present inside the vacuoles after 5 h in virus-free medium culture. On the other hand, I have never found virions free in the cy-

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toplasm outside the digestive vacuoles. Neither have I found similar structures as the so called factories, where these viruses replicate in sensitive cells.

During the first hour of the experiments the nuclei and the cytoplasm remain without changes. After 1 h, ultramicroscopic observations show in the cytoplasm some groups of vesicles in the proximity of the mitochondria (Pl. I 3). The profile of endoplasmic reticulum adjacent to the mitochondria has ribosomal granules, whereas the other side is smooth. The vesicles arise by budding from this part (Pl. II 4). These groups of structures are very numerous after 2 h and a half of culture inoculation appearing in the proximity of nearly all mitochondria (Pl. II 5). Later the regression of these vesicles was clearly observed. Plate III 6 shows their appearance in a cell 20 h after the beginning of the experiment. The cytoplasm regains its normal appearance when the viruses are eliminated. These groups of vesicles were also observed in the cytoplasm of early encysting individuals from virus inoculated cultures (Pl. III 7), but so far, we have never found these exstructures in excysted individuals coming from virus inoculated cultures.

Cells whose culture was inoculated once a week for 5 consecutive weeks do not contain these structures a week after the last inoculation.

Preliminary experiments with *Paramecium bursaria* show that vaccinia virus does not produce these kind of structures in the cytoplasm of these ciliates 2 h after culture inoculation. As observed at ultrastructural level (Pl. IV 8, 9), neither the symbiont chlorelles nor the structures of *P. bursaria* show a reaction against the virus, which seems to be eliminated in the food vacuoles without changes in the cell.

Discussion

The replication cycles of some poxvirus, among them vaccinia virus, are rapid; mature progeny are formed in sensible cells within 5 h from the onset of penetration (Dales 1973). In this way the hypertrophy of membranous cisternae in the cytoplasm of *O. acuminatus* 2 h after culture inoculation could be the start of infection, as happens in some sensitive cells (Amako and Dales 1967) where the proliferation of membranous cisternae occurs as a cytopathic response of L-cells to Mengovirus infection. Also after superinfection with human cytomegalovirus, an extensive synthesis of proteins was suggested by a marked proliferation of rough endoplasmic reticulum, polysomes and Golgi vesicles (Hung et al. 1948). Nevertheless, as described in results, cells of *O. acuminatus* do not contain these structures after the viruses have been eliminated; there are no viruses in the vacuoles of this ciliate 48 h after culture inoculation, and the ciliates divide, encyst and excyst normally, even after several culture inoculations.

On the basis of their morphology alone, these groups of saccules appear to be in *O. acuminatus* stacks of Golgi vesicles. Although the ultrastructure of vegetative cells and cells in encystment–excystment processes has been studied thoroughly in

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this ciliate (Jareño 1985), these groups of vesicles were observed only occasionally. These aggregations of small vesicles could represent a Golgi apparatus. Its structural variations would be in agreement with other observations in different kinds of cells (Waley 1975), concerning the quantitative and morphological changes of the Golgi apparatus when such cells are under different physiological conditions. It has been described that frequently there is a considerable increase in the number of stacks of Golgi cisternae just before the onset of the secretory phases (Claude 1970). Their hypertrophy in *O. acuminatus* could be a kind of reaction of the cytoplasm of this ciliate to eliminate or neutralize some substances released from the food vacuoles during early digestion of the viruses.

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EXPLANATIONS OF PLATES I–IV

1: Schematic design showing three major components of a sectioned vaccinia virus: lipoprotein membrane, core and lateral bodies (LB)

2: Vaccinia viruses at ultrastructural level as they appear in the food vacuoles of *O. acuminatus*. The majority of particles are dissolved, although there are some recognizable virions (arrows). ×92 000

3: Cell of *O. acuminatus* 1 h after culture inoculation by vaccinia virus. The virions (v) inside the digestive vacuole are in different phases of degradation. Arrow shows a cluster of membranous cisternae near the mitochondria. ×17 000

4, 5: Cells of *O. acuminatus* 2 h and a half after culture inoculation. In phot. 4 arrows show blebs, as explained in the text. In phot. 5 arrows show clusters of membranous cisternae. ×60 000, ×63 000

6: Aspect of the cell cytoplasm 20 h after culture inoculation. The group of vesicles is significantly smaller (arrow). ×46 000

7: Cytoplasm of encysting individual with membranous cisternae (arrows). See the ectocyst precursors (cp.) ×31 920

8, 9: Cytoplasm of *Paramecium bursaria* 2 h after culture inoculation by vaccinia virus. (dv) digestive vacuole. ×6840, ×26 000

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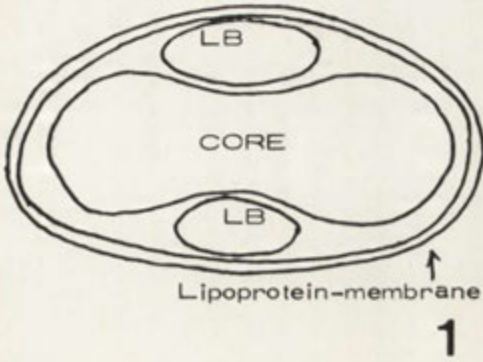

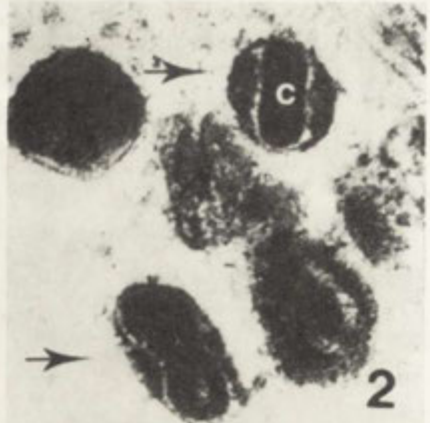

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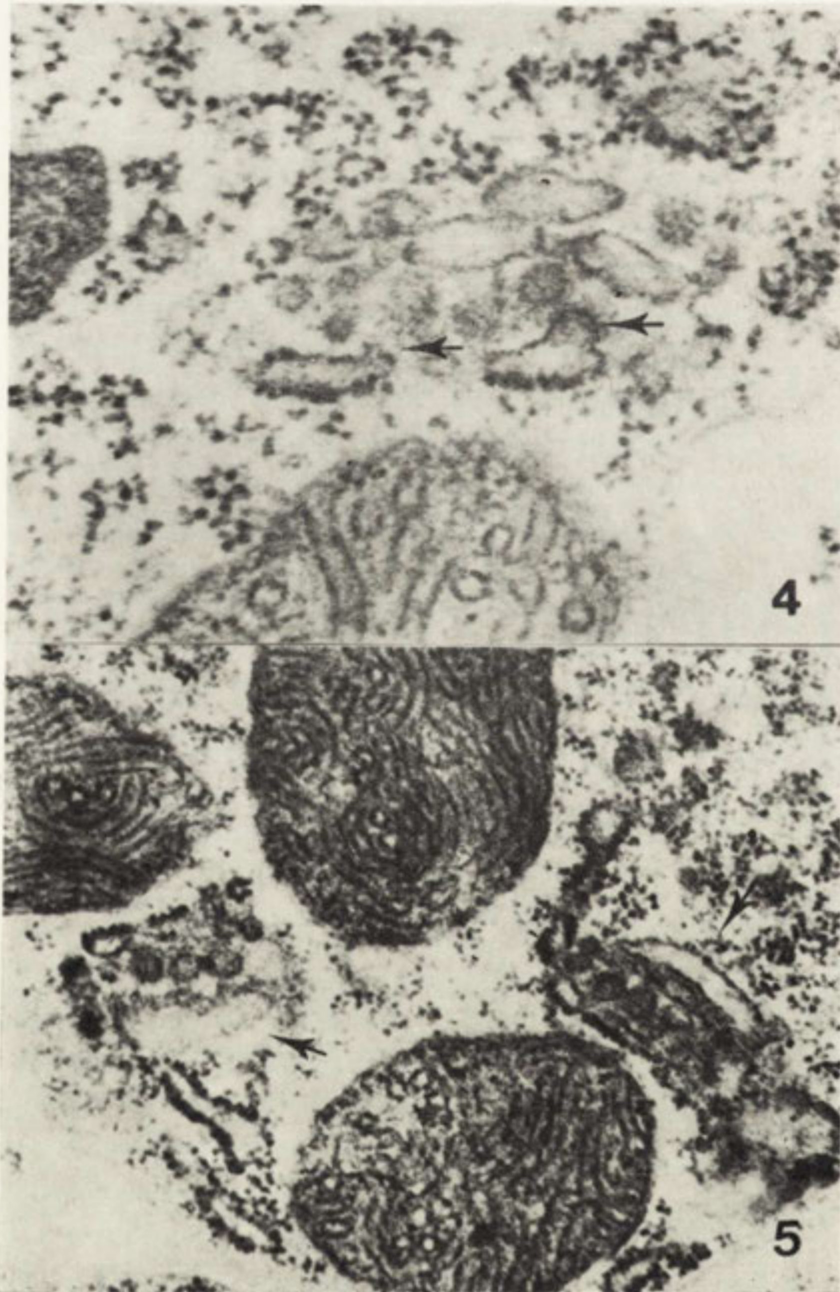

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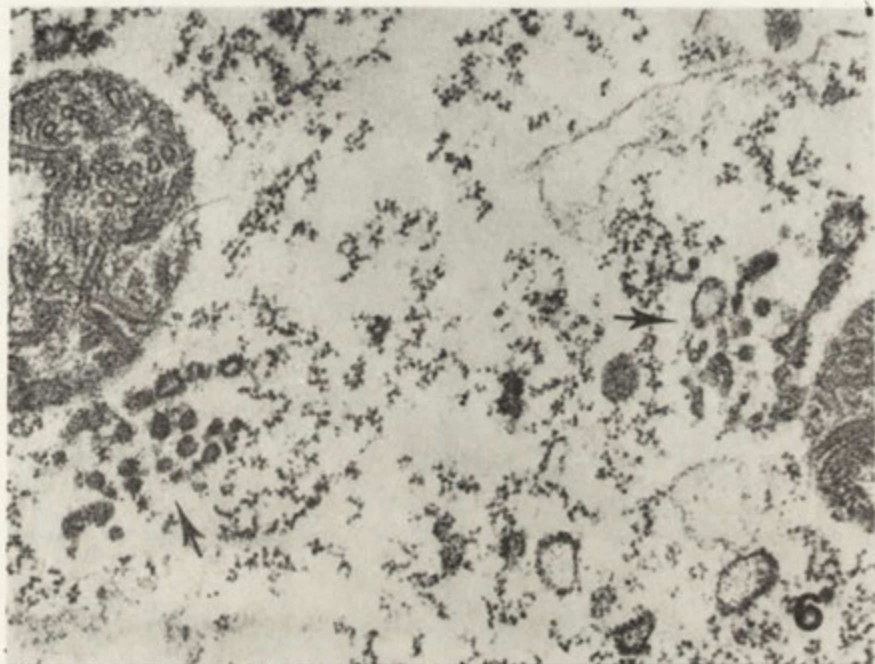

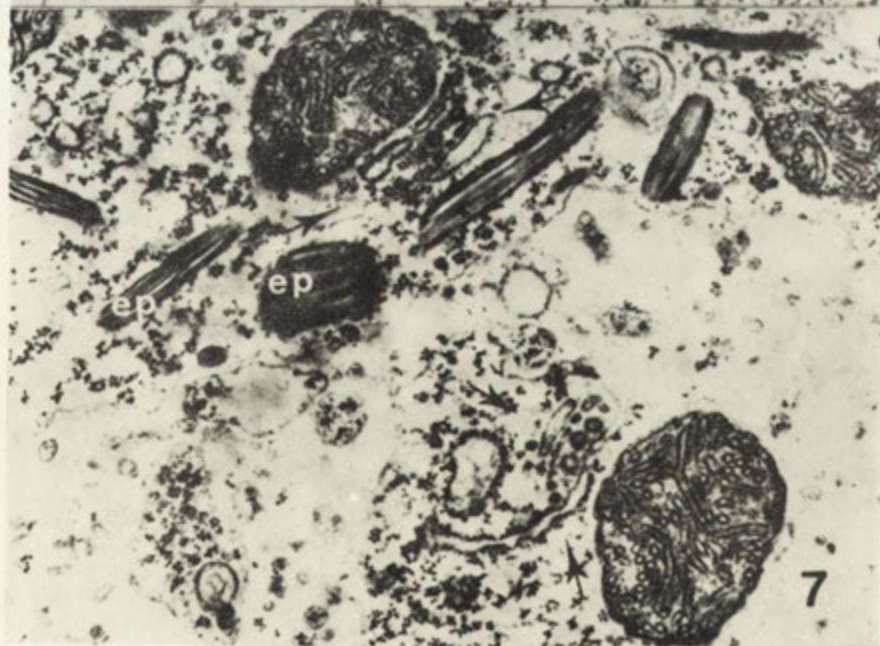

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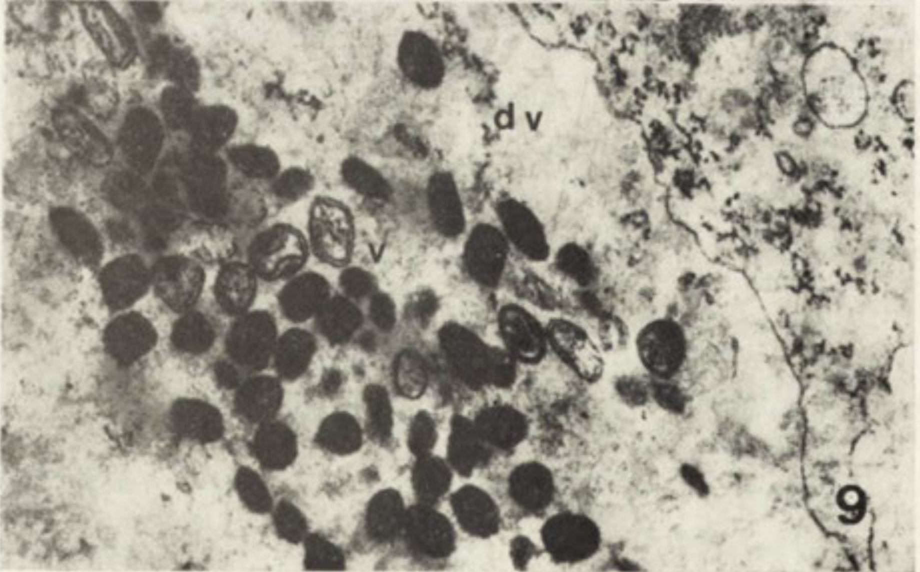

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Enkystement externe chez *Difflugia elegans* (*Rhizopoda*, *Testacea*)

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Synopsis. Dans cette note, nous décrivons un mode particulier d'enkystement externe chez le Rhizopode testacé *Difflugia elegans* Penard. La membrane du kyste étant protégée par une enveloppe constituée d'éléments quartzeux.

Si le phénomène d'enkystement est bien connu chez de nombreux genres de Thécamoebiens, il est beaucoup plus rare chez les *Difflugia* qui semble se contenter d'une certaine forme de prékyste. Penard (1902), cite le cas de *Difflugia pyriformis* dont le plasma jaunâtre et poussiereux était enroulé en boule; Nous avons souvent observé ce mode chez de nombreuses grandes espèces de ce genre.

Il est probable que l'enkystement véritable, n'est pas indispensable chez les espèces habitant les grands fonds, ou la sécheresse est moins probable, ce qui n'est pas le cas pour les espèces des biotopes soumis à de fréquentes dessications des petites mares et pièces d'eau de la plaine, dont la masse aquifère peut être fort variable au cours des saisons.

Dans le genre *Euglypha* par exemple, il est fréquent d'observer des kystes hyperprotégés entourés d'écailles siliceuses que l'on peut considérer comme des kystes de longue durée.

Nous décrivons ici un cas assez rare de formation d'un kyste externe chez *Difflugia elegans* Penard, 1890.

Cette espèce est fréquente dans le sapropèle des régions de la berge des étangs et surtout des petites mares.

Cinq variétés et une forme ont été décrites: v. *teres* Penard, 1899, v. *angustata* Deflandre, 1926, v. *bicornis* Jung, 1936, f. *tricornis* Jung, 1936, v. *lepida* Schönborn, 1966 et v. *parva* Chardez, 1969.

Les observations relatées dans cette note, concernent *D. elegans* dans sa forme typique.

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Matériel et méthode

La population de *D. elegans*, provient d'une petite mare de 4 m×5 m, peu profonde, entourée d'arbres (Sassor, Prov. de Liege Belgique).

Les échantillons ont été prélevés dans le sapropèle du fond, parmi les feuilles mortes en décomposition, pH = 7,9.

Environ 50% des individus étaient en état de formation d'un kyste externe recouvert d'éléments quartzeux.

Observations

Chez *Difflugia elegans* il n'est pas rare de rencontrer des individus dont le pseudostome est garni d'un bouquet de particules pierreuses agglutinées comme l'a bien décrit Penard (1890, 1902). Ces bouquets seraient d'après Rhumbler (1891) une provision d'éléments destinés a former une nouvelle thèque. Il est probable comme l'écrivait Penard que certains de ces éléments pierreux proviennent du cytoplasme de l'individu, oú ils ont séjourné quelques temps.

D'après nos observations, ces matériaux de réserve poussés vers l'extérieure servent en réalité à la construction d'une coquille sphérique, qui, une fois terminée sera destinée à protéger un kyste (Fig. 1 *1*).

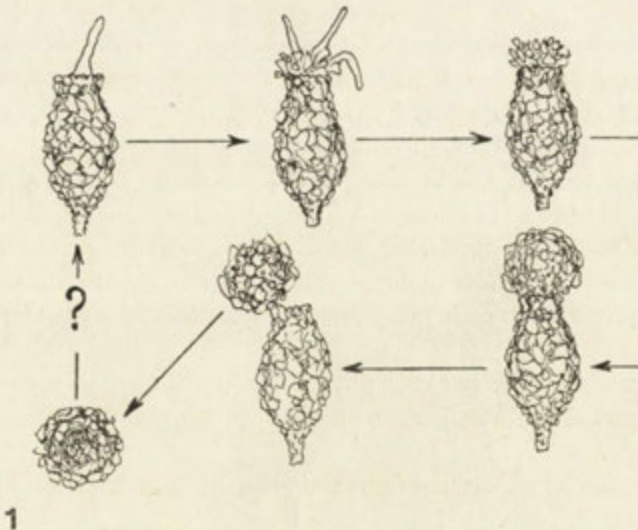

Fig. 1 *1*. Représentation schématique de la formation d'un kyste externe chez *Difflugia elegans*

Dans les préparations, la présence d'une bulle d'air dans cette enveloppe pierreuse, semble démontrer que celle-ci reste un certain temps vide avant l'enkystement proprement dit (Pl. I a–d).

Progressivement ces éléments pierreux prennent la forme sphérique, ensuite le Rhizopode passe à l'intérieur pour s'enkyster.

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Une fois le kyste formé, il reste accolé au pseudostome de la thèque mère un temps variable, il est probable qu'il s'en détache par l'effet du hazard, car il abandone toujours une thèque vide. Le diamètre extérieur de ces kystes externes varie de 38 à 45 μm.

Le dékystement et le retour à la forme normale nous reste inconnu... La présence dans nos prélèvements de nombreux éléments pierreux sphériques (Pl. I h—J) assimilables à première vue au groupe des „Globuleuses", semble démontrer qu'il s'agit dans ce cas particulier de formation de kystes longue durée, qui, comme le montre (Pl. I j) une fois débarassés du revêtement pierreux externe par compression à l'aide d'une micro-spatule, montre une membrane épaisse, doublée d'une zone interne hyaline entourant un cytoplasme granuleux.

La grande difficulté d'obtenir des élevages „in vitro" de certaines espèces de Thécamoebiens dont la thèque est contituée d'éléments exogènes permet difficilement de suivre la totalité de leur cycle biologique.

Enfin, signalons que nous avions déjà observé ce cas d'enkysteme chez *D. elegans* provenant de prélèvements fait sur les berges d'un grand étang peu profond (Eplatis, Ardenne belge).

Conclusion

La formation de kystes externes correspond à un mode particulier déjà signalé chez quelques espèces de Thécamoebiens: *Difflugia bicuspidata* Rhumbler par Rhumbler (1898), *Sphenoderia lenta* Schlumberger par Awerintzew (1906), *Difflugia* sp. par Schönborn (1975), *Valkanovia delicatula* Valkanov (original.)

Ce mode d'enkystement assez rarement signalé ne semble pas en rapport avec la reproduction de l'espèce. La présence d'une membrane kystique protégée par une enveloppe quartzeuse solide, semble démontrer qu'il s'agit de kystes de résistance longue durée.

SUMMARY

In this note, we describe a particular way of external encysting by the Testate Amoebae *Difflugia elegans* Penard.

The cyst limb being protected by an envelope made of quartz elements.

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EXPLICATION DE LA PLANCHE I

Pl. I 2: *Difflugia elegans*
a–e, g: formation de kystes (×400)
f: thèque vide et thèque avec kyste (×400)
h: kyste isolé (×400)
i: kyste isolé (×600)
j: kyste débarrassé de son enveloppe pierreuse par micromanipulation. La flèche indique la membrane kystique partiellement éclatée (×600)
k: variété plus grande et plus globuleuse présente dans nos prélèvements (×400)

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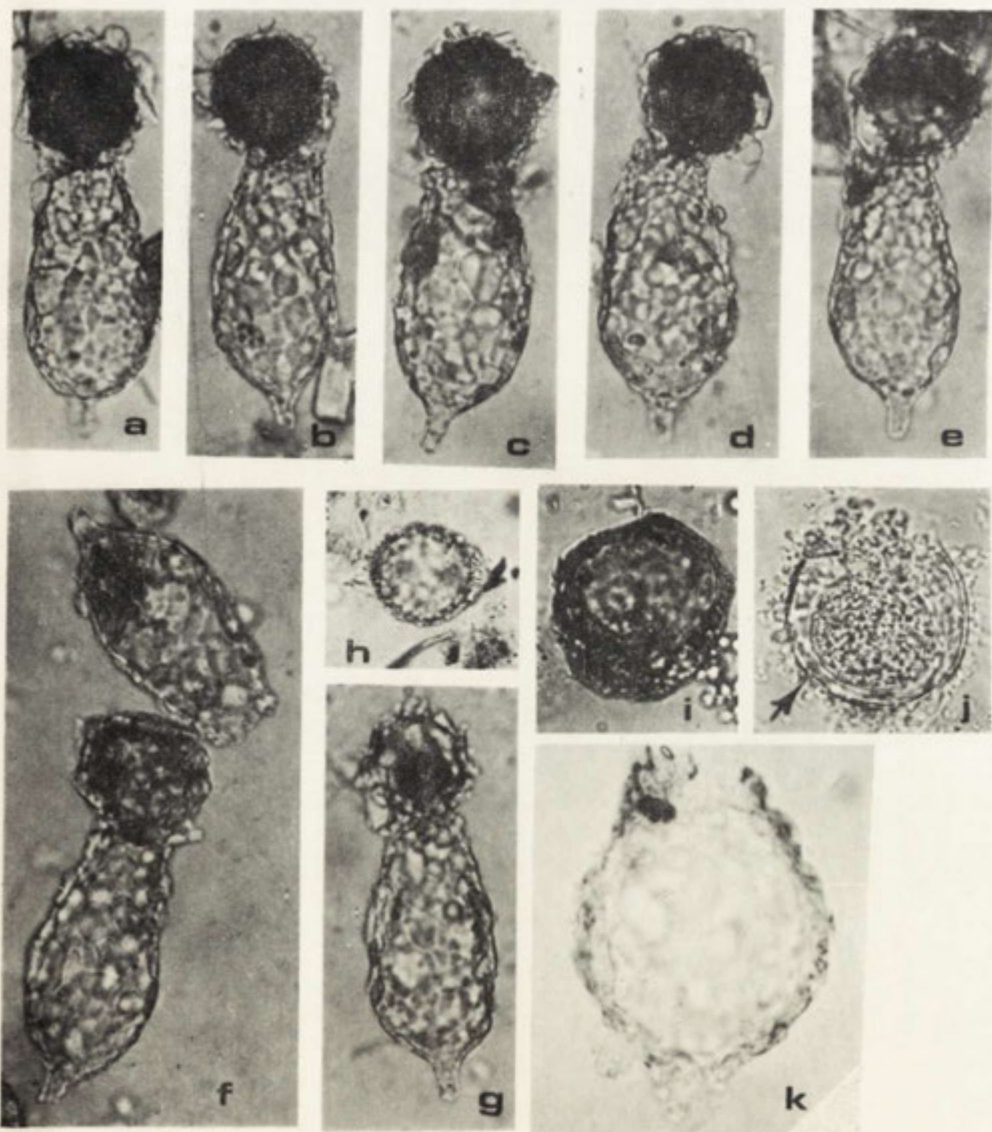

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Relationship between the Size of Cell and the Number of Its Ciliary Rows in the Ciliate *Dileptus*

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Synopsis. Dileptus, like the majority of other ciliates, has its somatic ciliature patterned into longitudinal rows, termed the kineties. In this study the relationship between the cell size and the number of kineties was examined, using four groups of cells differing in size. A very large overfed cells, and very small cells obtained from regenerated fragments were studied, and compared to control cells. Variability in the number of kineties is very high in control cells, indicating that both addition and deletion of ciliary rows takes place in *Dileptus*.

A confirmation of the upward regulation in the number of kineties has been found in this study. There are more kineties on the large bodies of overfed cells (after 4 days of overfeeding) than on control cells. The number of kineties seems to increase slower than the circumference of cell body, resulting in the widening of interkinetal space, and the change of kinetal pattern from longitudinal to oblique against the long axis of the cell.

To explore the possibility of the downward regulation of the number of kineties, two groups of small fragments were studied 24 h after the operation. These two groups differed in the initial number of somatic kineties. It appeared that regenerated tiny dileptuses still have all the kineties they originally possessed. It was concluded that the downward regulation in the number of kineties takes more than 24 h.

An adjustment of ciliary pattern to experimentally changed size of the ciliate is best documented in *Paraurostyla* (Jerka-Dziadosz 1976, 1977, Bąkowska 1980, 1981, Bąkowska and Jerka-Dziadosz 1980). Tiny cells, diminished by cutting or starvation, develop fewer cirri, and composed of fewer basal bodies. The large cirri present in the cell before the operation or starvation are resorbed in due course of the morphogenetic process. Holotrichous ciliates, like *Tetrahymena* or *Dileptus*, do not resorb somatic ciliature during morphogenesis. The regulation of the number of kineties must rely on an addition or destruction of some of the somatic kineties, leaving all the rest of ciliary rows intact.

It is known that both the addition and deletion of kineties are taking place in *Tetrahymena* (Nanney 1967, 1968, Frankel 1980). The change in the number of kineties is influenced by environmental conditions (Nanney 1966). It has been found

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that there exists a stability range in the number of kineties, i.e., some corticotypes are less likely to undergo change in the number of kineties than the others, moreover the corticotypes above stability range are rather losing the kineties, those below the range are inclined to form new kineties. It is not clear whether the naturally occurring variations in kinety number follow the appropriate changes in the cell size of *Tetrahymena*.

The aim of this study was to evoke a change in the number of somatic kineties in *Dileptus*, by drastically changing the size of cell. The regenerative capacities of *Dileptus* allow to obtain very small viable fragments. It is also possible to enlarge dileptuses by overfeeding (Golińska 1986). Observations on the variation in the number of kineties indicate that there exists a cell-size-dependent regulation of the number of somatic kineties, and that the regulation is a very slow process, taking more than 24 h.

Materials and Methods

Dileptus used in this study is *Dileptus margaritifer*, previously referred to as *Dileptus anser* (see revision by Wirnsberger et al. 1984). Culture methods are described elsewhere (Golińska and Jerka-Dziadosz 1973), as well as the methods to obtain large overfed cells (Golińska 1986). Stock cultures were kept in deep glass vessels and fed every other day with *Colpidium* sp.

Four groups of cells of different size were studied. The first group (further referred to as group 1) were control cells taken from stock culture. The second group were large overfed cells (group 2), obtained by keeping cells in a constant presence of food for 4 or 5 days. The third group (group 3) were dileptuses regenerated from the anterior long process of the cell, termed proboscis. Somatic kineties on the proboscis are less numerous than on the middle portion of the cell, thus the initial number of kineties in these fragments was low. Group 4 consisted of small fragments cut off from the middle portion of the cell, thus the initial number of their kineties was the same as in group 1.

Transections were made by hand, using a microknife. Cells were cut on Petri dishes, and fragments were isolated into depression slides. The medium used was that taken from stock cultures deprived of food. Groups 3 and 4 were fixed 24 h after the isolation.

Measurements were made, and somatic kineties were counted on preparations impregnated with protargol after the modified method of Dragesco (Golińska 1984). The size of cells was estimated by their length and width. For the length of cell only the length of trunk was measured, while the narrow anteriorly located process called the proboscis, was omitted from the calculations. This was because the proboscis is always more or less twisted on preparations, which makes measurement difficult. The circumference of the cell trunk was calculated from the width of the cell, using the formula for the circle perimeter in groups 1, 3 and 4. For group 2, because these large cells were usually flattened on preparations, the depth of the trunk as well as its width was measured, and the circumference was calculated using the formula for the ellipse perimeter. Both the circumference of the cell trunk and the number of kineties were measured in the middle portion of the trunk. This is the place where the highest number of somatic kineties may be encountered, since not all of them reach the posterior and anterior poles of the cell. The number of kineties was estimated by measuring the distance between 6 kineties, then the trunk perimeter was divided by this distance value, finally multiplication by 5 gave us the number of kineties.

Statistical analyses were made by Data Computing Center of the Nencki Institute. A comparison of means was made using Duncan's multiple range test, and to estimate relations between different features, Spearman's coefficient of rank correlation was t-tested for significance.

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Results

The cell of *Dileptus* consists of the trunk and of the anteriorly located process called proboscis (Fig. 1). The ciliature falls into two main categories: oral and somatic. The oral ciliature encircles the cytostome situated at the basis of the proboscis, and occupies the ventral part of the proboscis. The somatic ciliature is represented by locomotor and sensory cilia. The sensory cilia occupy only the dorsal side of the proboscis. Both locomotor and sensory cilia are patterned into longitudinal rows called kineties, and it was the number of locomotor kineties that was estimated in this study. The locomotor cilia occupy the whole surface of the trunk and can also be found on both sides of the proboscis. On the dorsal side several locomotor kineties of the trunk are anteriorly prolonged into rows of sensory cilia on the proboscis. On the ventral part many locomotor kineties of the trunk end at the margin of a cytostomal field (for details see Grain and Golińska 1969). The posterior portion of the trunk is tapered into a narrow tail, and only a few kineties reach the tip of the tail. The locomotor kineties end one by one in the posterior narrowing part of the trunk. Thus the locomotor kineties are most numerous in the middle portion of the trunk, and this is the place where their number was counted.

The length and perimeter of the trunk, as well as the spacing of somatic kineties,

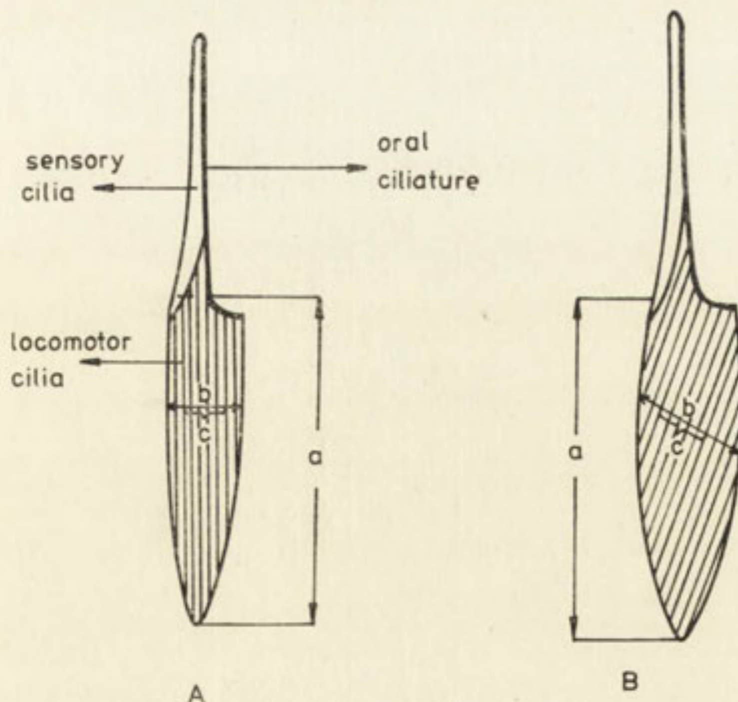

Fig. 1. Pattern of somatic kineties on lateral side of *Dileptus*. A — control cell, B — overfed cell, a — trunk length, b — trunk diameter, c — interkinetal distances used for calculations. Note the oblique orientation of kineties in overfed cells

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Table 1

Cell size, number and spacing of kineties in four group of *Dileptus* cells. Data are given ±S.D.

Specification	Length of trunk (μm)		Circumference of trunk (μm)		Interkinetal distance (μm)		Number of kineties	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Group 1	242.62±74.74	101.50–380.10	150.80±17.35	121.45–184.00	3.13±0.40	2.36–4.21	48.28±6.61	36–61
Group 2	204.54±32.41	149.10–280.00	242.90±24.92	198.06–295.24	4.27±0.43	3.64–5.71	57.02±6.81	46–69
Group 3	76.51±20.44	46.20–121.80	85.61±9.78	67.26–112.10	2.59±0.30	2.07–3.64	32.91±3.89	24–41
Group 4	96.69±24.08	51.80–157.80	137.23±26.99	72.61–195.06	2.98±0.59	1.43–4.00	46.51±5.92	38–61

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were measured using the four groups of cells differing in size. The length of trunks was measured from the base of the proboscis to the end of the tail (Fig. 1). The circumference of the trunk was measured in its middle part. The number of kineties was calculated by measuring the interkinetal distance, and then the cell perimeter was divided by this distance. The results are presented in Table 1.

Group 1 contained cells taken from growing culture, thus the cells were in all stages of cell cycle (except for late fission, since these were not taken into account). This is reflected in a very high variability of cell length: the shortest cell is almost three times shorter than the longest one. The cell perimeter varies within much narrower range, and so does the number of kineties.

Group 2 contains large, overfed cells. Their trunks are somewhat shorter than in group 1 (84% of the trunk length in controls), but trunk perimeter is 162% of that found in group 1. The number of kineties in group 2 is increased in comparison to group 1, but the increase is not proportional to the increase in trunk circumference (only 119%). There is a substantial increase in the interkinetal distance (142%). The kineties on the large trunks of overfed cells are oriented not quite parallel to the long axis of the cell (Fig. 1 B, Pl. I 3). This reminds the twisted kineties in contracted ciliates like *Spirostomum* or *Stentor*, when the increase in cell width and the shortening of its long axis is accompanied by an oblique orientation of kineties. This might mean that in overfed dileptuses both higher numbers of kineties and larger interkinetal distances are not sufficiently increased to allow the proper longitudinal orientation of ciliary rows. The width of overfed cells was measured at a right angle to ciliary rows, and so were measured the interkinetal distances (Fig. 1 B). Thus the calculated circumference of trunks in this group may be slightly higher than it really is.

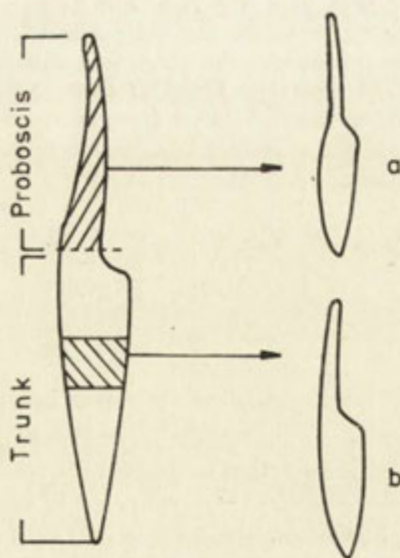

Fig. 2. A normal cell is shown (left) with dashed areas which were cut off to obtain small cells: a — group 3, b — group 4

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Group 3 contains tiny dileptuses regenerated from cut off probosces, fixed 24 h after the operation (Fig. 2, Pl. I 4). Since the proboscis contains much less locomotor kineties than the trunk does, the initial number of kineties was rather low in these fragments (the fate and formation of locomotor kineties on the severed probosces are presently under investigation). When tiny dileptuses in group 3 are compared to the control cells (Table 1), their trunks are 31.5% of the normal length, 56.8% of the normal circumference, while the interkinetal distance is 82.7% of the normal one, and number of kineties is 68.1% of the value found for group 1. Thus the spacing of kineties is the feature most resembling that in the control cells.

Group 4 consists of tiny dileptuses regenerated from the pieces cut off from the middle of normal cells trunks. Thus their initial number of kineties was the same as in group 1, while the cell volume was considerably diminished (Fig. 2). Twenty four hours after the operation dileptuses in group 4, when compared to those in group 1, had trunk measurements amounting to 39.8% of the normal length and 91% of the normal perimeter. The trunks were covered with 96.3% of the normal number of kineties, interkinetal distance being 96.1% of the normal distance. Thus there is no reduction in the mean circumference of cells, as well as in the mean number and spacing of locomotor kineties during the first 24 h after the operation. The only sign of a possible future reduction in kinety number is an unusually wide range of cell perimeter and of interkinetal distances.

For a better comparison of data obtained in each group of cells, the Spearman's coefficient of rank correlation was t-tested for significance. The results are presented in Table 2. It has been found that there is no correlation when the number of kineties is compared to cell length within each group of cells. The number of kineties and their spacing was found to be correlated with cell perimeter in all groups studied, except group 4. The correlation coefficient for relationship between the kinety number and cell perimeter was 0.53 in group 1, 0.69 in group 2, 0.58 in group 3, and 0.29 in group 4. This lack of correlation in group 4, in spite of the maintenance of mean values similar to those in group 1, must be due to the already mentioned widening of the range of cell perimeters and interkinetal distances in group 4.

Table 2

t-test for significance for rank correlation between: A — number of kineties and circumference of trunk, B — number of kineties and interkinetal distance, C — number of kineties and length of trunk, NS — non significant, p — level of significance

Specification	A	B	C
Group 1	$p < 0.01$	$p < 0.001$	NS
Group 2	$p < 0.001$	$p < 0.01$	NS
Group 3	$p < 0.001$	$p < 0.01$	NS
Group 4	NS	NS	NS

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To compare mean values obtained for cell size and number of kineties in different groups of cells, Duncan's multiple range test was used. Results are presented in Table 3. It appears that the length of trunks is practically the same for groups 3 and 4, and that group 4 has the same number and spacing of kineties as found in

Table 3

Comparison of mean values obtained for different groups of cells. Duncan's test. p — level of significance, NS — non significant

Specification	Groups 1 and 2	Groups 1 and 3	Groups 1 and 4	Groups 2 and 3	Groups 2 and 4	Groups 3 and 4
Length of trunk	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
Circumference of trunk	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < p.001$
Interkinetal distance	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.01$
Number of kineties	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$	$\rho < 0.001$	$p < 0.001$

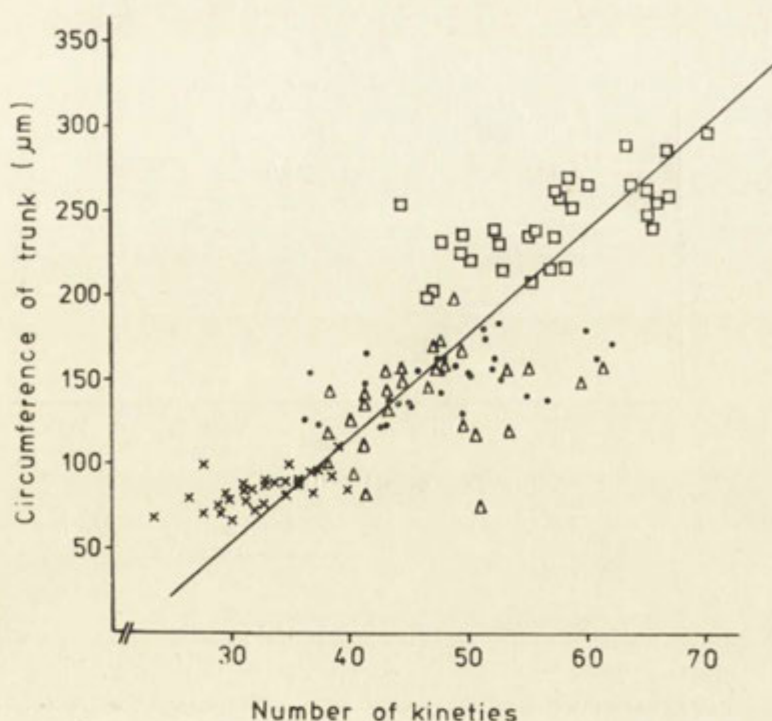

Fig. 3. Relationship between number of kineties and circumference of trunk in all cells studied. Black dots — group 1, squares — group 2, crosses — group 3, triangles — group 4

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group 1. This again indicates that dileptuses in group 4 maintain their initial number of kineties during 24 h after the operation. It is interesting that the cell perimeter is changed in group 4 in comparison to group 1 ($p < 5\%$), its range is widened both towards the slightly wider cells and towards the very narrow ones (Table 1). This may indicate that the regulation of cell shape precedes the regulation of the number of kineties.

Finally, the relationship between the cell size and the number of kineties was estimated when the cells from all groups were jointly considered. The relationship between the number of kineties and the circumference of trunk showed the highest correlation (Fig. 3), with correlation coefficient 0.83. The number of kineties is also correlated with interkinetal distance, with coefficient 0.46; thus the smallest cells have the most crowded kineties, and the interkinetal space tends to be wider in large cells with numerous kineties (Fig. 4). Surprisingly, the number of kineties

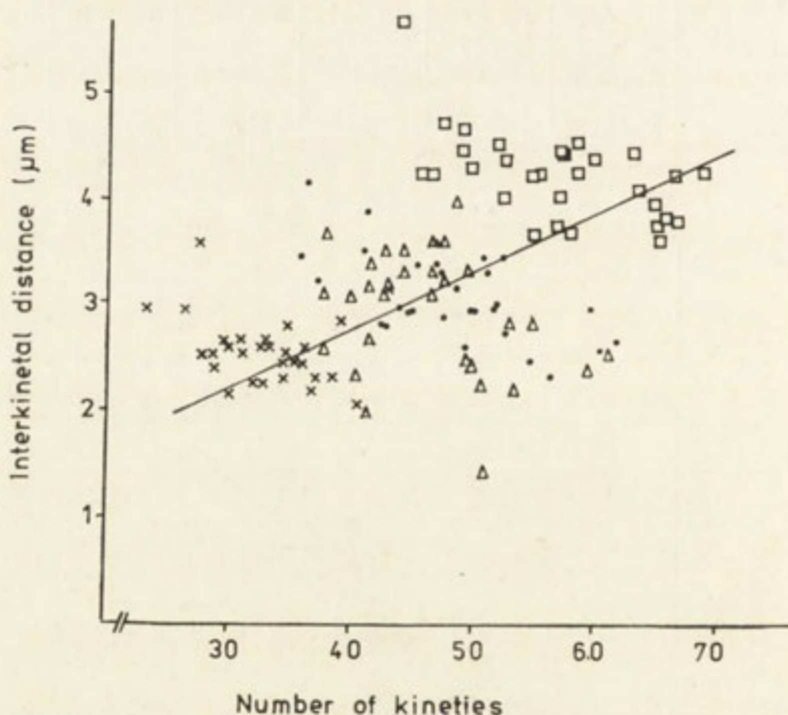

Fig. 4. Relationship between number of kineties and interkinetal distances in all cells studied. Black dots — group 1, squares — group 2, crosses — group 3, triangles — group 4

is correlated with the length of trunk (Fig. 5, correlation coefficient 0.50), while there was no correlation when each group was considered separately. Thus the number of kineties is correlated with cell length only in the case of extremely high variation of the cell size. It appears that in *Dileptus* the number of locomotor kineties is correlated with the size of trunk, especially with its circumference.

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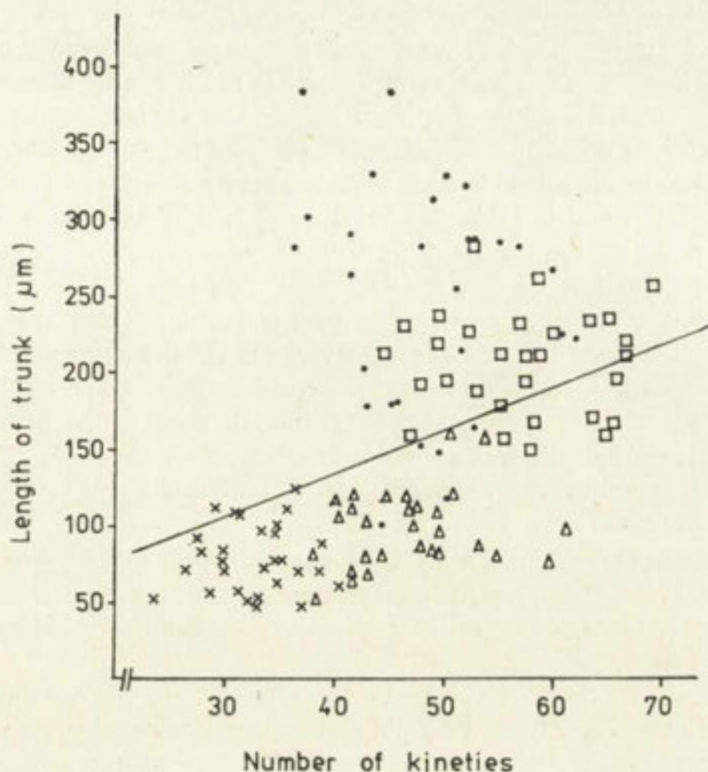

Fig. 5. Relationship between number of kineties and length of trunk in all cells studied. Black dots — group 1, squares — group 2, crosses — group 3, triangles — group 4

Discussion

The data obtained in this study have revealed several interesting features of pattern regulation in *Dileptus*. Firstly, there is a very high natural variability in the number of ciliary rows. This may not be unusual among holotrichous ciliates, since *Tetrahymena* when in stock culture has 15 to 35 kineties (Nanney 1966). Thus the largest cell has twice the number of kineties of the smallest cell, exactly like *Dileptus* with its range of kineties from 36 to 61. This variability is not related to the cell cycle. Cloned tetrahymenas show rather surprising stability in corticotype, with only occasional addition or deletion of a ciliary row (Nanney 1967, 1968, Frankel 1980). The variability in *Dileptus* can be ascribed to changes in cell volume resulting from the availability of food, since the number of kineties was found to be correlated with cell perimeter and not with cell length, the length undergoing fluctuations from fission to fission. The increase in the number of kineties is not proportional to the increase in the cell perimeter. There is also an increase in interkinetal space, which shows correlation with the circumference of the cell.

Secondly, it has been found that the regulation of the number of kineties follows an increase in cell size. This upward regulation occurs in overfed cells, sometimes

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during 4 days of overfeeding. The mean number of kineties increases by 10 in comparison to controls and the ranges for both groups are only partially overlapping. Again, the increase in the number of kineties is much smaller than the increase in cell perimeter, and interkinetal distances become larger in large cells. Very interesting is the oblique orientation of kineties in many of the overfed cells. It looks as if a maximum of the interkinetal distance has been reached, and further enlargement of the cell has evoked this slant in the orientation of ciliary rows. This supposition will be checked in further study.

The third question, whether the downward regulation of kinety number may exist in *Dileptus*, cannot be answered on the basis of data obtained in this study. Dileptuses diminished by cutting probably maintain all the kineties which they initially possessed. A decrease in the number of kineties can take more than 24 h, and small cells were not allowed to live long enough to regulate their pattern. Twenty four hours is more than enough for fragments to regenerate all the excised parts. Oral structures are known to regenerate in 3 h (Golińska and Grain 1969), regeneration of the tail takes about 2 h (Golińska 1974). The recovery of cell shape, with a proper proboscis to trunk length ratio, is known to take more than 24 h (Golińska 1979). It seems that the regulation of the number of kineties falls in the category of restoration of cell proportions, rather than in the category of regeneration processes.

In ciliates other than *Dileptus* the ways of regulation of kinety number are also almost totally obscure. Several possibilities of kinety addition are known. A kinety may be "grafted" into the cortex due to imperfections in exconjugant separation (Beisson and Sonneborn 1965), a disrupted kinety may not heal properly and new ciliary rows are formed (Kaczanowska 1975), and a new kinety may form as a branch of oral primordium during rapid swimmer transformation in *Tetrahymena* (Nelsen and Frankel 1979). Only in the last case the change of kinety number may have a regulatory character, since it is related to the change in cell volume and surface area. We hope that further studies on *Dileptus* may reveal more details concerning the origin, formation, and destruction of the kinety during pattern regulation.

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EXPLANATION OF PLATE I

1: Somatic kineties on the trunk of control cell (group 1). Arrows point to several basal bodies belonging to one kinety. ×2800
2: Somatic kineties on the trunk of a group 4 cell. Arrow (c) indicates cilium. ×2800
3: Oblique orientation of kineties on the trunk of overfed cell (group 2). ×1300
4: Somatic kineties on trunk of a small group 3 cell. ×2800

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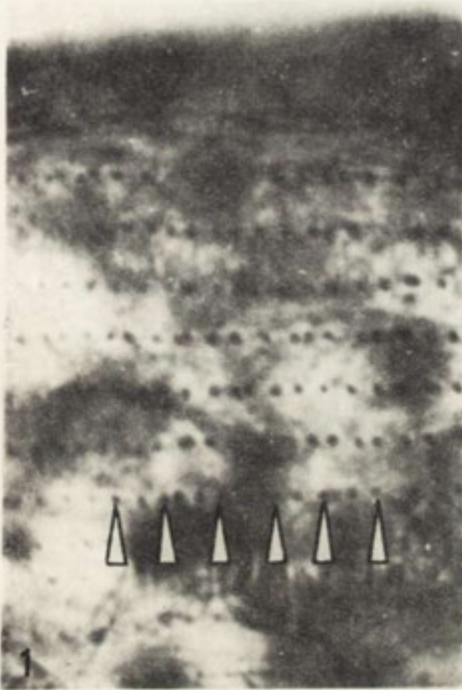

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Motor Behaviour of *Amoeba proteus* during Glutaraldehyde Fixation

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Synopsis. The glutaraldehyde + polyethylene glycol fixative, recommended to study the differentiations of contractile structures in *Amoeba proteus* related to locomotion, provokes well defined motor responses during the fixation procedure, expressed mainly in the reversal of endoplasmic streaming and retraction of all advancing fronts. It may improve the picture of the motile apparatus by reinforcing contraction and inducing it even in formerly expanding cell regions. It seems, therefore, that any conclusions about the original conformation and topography of the contractile cell layer during normal locomotion based on this method are subject to caution, unless confirmed by other methods, in particular by *in vivo* techniques.

Topography of ultrastructural differentiations in the motile apparatus of *Amoeba proteus* during locomotion may be an efficient tool for elucidating some still obscure aspects of amoeboid movement. But the task is extremely delicate because of the great lability of the contractile layer of giant amoebae, which is expressed by its continuous disintegration in the rear regions of moving cell and permanent reconstruction in the front of locomotion, and by the parallel transformations easily produced by all kinds of contracting or relaxing external stimuli. Therefore, an almost instantaneous fixation of the actomyosin system is the necessary precondition of any ultrastructural research conceived as a means to study the physiology of movement. Otherwise, the reliability of results may be subject to caution, because the electron microscope pictures produced may not exactly correspond to that experimental situation and that functional state of the motile apparatus which had to be investigated.

Stockem et al. (1982) tested four fixating solutions, each based on 1–3% glutaraldehyde but differing by addition of polyethylene glycol, osmium tetroxide, picric acid or phalloidin. They concluded that the contractile system of amoebae is better visualized by glutaraldehyde + polyethylene glycol mixture, than by the fixative containing osmium tetroxide, and the effects may be still improved by addition of phalloidin at the initial pre-fixation stage. The new fixating solutions yield-

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ed the results interpreted by the authors as being partly controversial to some earlier data, in particular those concerning the structure of the front and the tail region of amoeba. It became therefore desirable to check in what extent the recommended solutions satisfy the requirement of instantaneous fixation, as formulated above.

The experiments described in the present paper were completed in 1983. They were based on the cinematographic frame-by-frame analysis of the course of fixation of moving *Amoeba proteus* by slow and rapid, glutaraldehyde- and/or osmium-based fixatives. A preliminary report of the results has been presented in July 1984 to the 1st Polish Conference on Cell Biology (Grębecka et al. 1985).

Material and Methods

Three following fixating solutions were used:

(1) 2% glutaraldehyde with 2% polyethylene glycol, in 0.15 M PIPES buffer (after Stockem et al. 1982).

(2) 2.5% glutaraldehyde with 1% OsO_4, in 0.15 M PIPES or 0.1 M cacodylate buffer,

(3) The same solution as (2), but with addition of 0.1% acrolein.

The solutions (1) and (2) were used to compare the motor behaviour of amoebae.

The material was taken from mass cultures of *Amoeba proteus* (Princeton strain) grown in the Pringsheim medium at room temperature, and fed twice a week on *Tetrahymena pyriformis*. The well attached, locomotory active cells were used for experiments, 2—3 days after feeding. After the transfer with a drop of culture medium to an open slide, they were left in moist chamber for 10—15 min to re-attach to the glass surface and to recover the usual motor behavior.

The slides with amoebae (without cover slips) were then placed on the microscope stage. Fixation was run under continuous visual control of individual cells and either photomicrographic or cinematographic pictures were simultaneously taken. The fixatives 1 or 2 were added in a largely excessive amount, 3—5 times the volume of the drop with amoebae. Observations and recording were started about 1 min before adding the fixating solution and came to the end after a complete standstill of the treated specimen. Photomicrographs were exposed at varying intervals with a Robot camera for taking serial pictures. Cinematographic films (16 mm) were run frame-by-frame, at the speed of 1 frame per second, by a combined Bolex-Robot equipment. The microscope was equipped with the differential interference contrast optics of Pluta system (PZO — Warsaw).

For the electron microscopy amoebae were, after fixation in the solutions (2) or (3), dehydrated in graded ethanol concentrations followed by propylene oxide, and embedded in Epon 812. Fine sections were cut on LKB microtome and examined in JEM 100B electron microscope at 80 kVh.

All the fixating solutions were freshly made and kept on ice before use.

Results

The polytactic amoebae fixed in the glutaraldehyde + polyethylene glycol solution have an apparently normal shape and their body configuration seems to be likely the same as it was before fixation. But the cinematographic records demonstrate at the first sight that this impression is fallacious. That becomes evident when the last frame taken ≤ 1 s before the contact of amoeba with the fixative (Pl. I 1, the black contour) and a frame exposed at the end of fixation procedure (the white

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profile) are plotted one onto another. In general the cells decrease in volume during fixation. Their frontal pseudopodia, which were advancing, are slightly but distinctly retracted. Other cell regions diminish in size and the uroid is manifestly withdrawn in the former direction of locomotion. It seems therefore obvious that, during 2–3 min which are needed to this type of fixative to operate, amoeba still has the possibility to display further motor responses which may be either opposed (retraction of the fronts) or parallel (withdrawal of the tail) to the original orientation of movement.

These motor responses, which continue during the fixation procedure or are elicited by contact with the fixative, were studied by frame-by-frame analysis of cinematographic films (Pl. I 2) and recorded photographically (Pl. II 3–5). Already at the moment of the first contact of the cell with the glutaraldehyde + polyethylene glycol mixture the direction of endoplasmic streaming is reversed (Pl. I 2, first fixation stage and Pl. II 3, a, b). The endoplasm is now flowing from the former fronts to the former tail, along the whole cell length, with the velocity which seems to be normal or even higher than usually. This response, which is specifically provoked by the fixative, is invariably produced in all individuals treated with glutaraldehyde and lasts usually 30–50 s. It is opposed to the former locomotory orientation of the investigated cell and thus it obviously must disturb the picture of morphodynamic differentiations between the advancing and withdrawing body regions. As a matter of fact, at this stage the frontal pseudopodia become always slender and withdraw (Pl. I 2). At their distal ends the hyaline caps disappear and a thin ectoplasmic layer (which normally is absent) appears beneath the outer membrane of pseudopodial tips (Pl. II 4). During the streaming reversal period the posterior body end of amoeba may either be still withdrawn (Pl. I 2, a), or kept more or less stationary (Pl. I 2, b), but the inflow of endoplasm into the tail region provokes its dilatation (Pl. II 5).

At the next stage, lasting from 1 to 2.5 min, the endoplasmic streaming resumes its original direction (Pl. I 2, the last fixation stage and Pl. II 3, c). It is accompanied by an intense retraction of the tail region which looks not different from its withdrawal during normal locomotion. However, in most cases (Pl. I 2, a and Pl. II 5, b) it remains inflated up to the end, and is fixed in that form. In the frontal cell region, the recurrent inflow of the endoplasm into the former advancing pseudopodia fails to reanimate the progression of their tips. They do not extend again and very often continue to shorten, but instead they grow in diameter (Pl. I 2, the last fixation stage and Pl. II 3, c). All these deformations mean that the renormalization of flow direction at the second and last stage of fixation is incapable to restore the state the cell had at the moment of its first contact with the fixative.

The monotactic amoebae, which are headed by vesicular frontal caps (Korohoda and Stockem 1976, Grębecka 1978, Hrebenda and Grębecka 1978) and are characterized by the direction of flow so stable that they fail to respond to the behavioural stimuli (Grębecki and Grębecka 1978), nevertheless respond in the same way to the glutaraldehyde + polyethylene glycol solution. The endoplasmic streaming in such cells is also reversed, and the front-tail effects

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of reversal are even more pronounced than in the polytactic forms, probably because of the simpler bipolar configuration of monotactic individuals (Pl. II 6). Their fixation with the glutaraldehyde and polyethylene glycol leads always to very strong dilatation of the tail region. A constriction, which indicates contraction, appears just behind the frontal cap vesicle, and the cap itself is partly or completely collapsing (Pl. II 7).

The motor effects described above are not due to the presence of the polyethylene glycol in the fixating mixture, because they are manifested as well in its absence, in the pure glutaraldehyde solution.

The situation changes diametrically when 1% OsO_4 is added to the glutaraldehyde solution. Instead of 2—3 min, the fixation takes less than 1 s. The results of cinematographic analysis shown in Pl. III 8, a, prove that as well the endoplasmic streaming as the ectoplasmic movements are stopped almost immediately. The cell does not change its size, shape or position. The light microscope pictures (Pl. IV 9) demonstrate also that its macrostructure remains unaltered. Even in a case of a defectuous fixation (the temperature effects) the cell is stabilized within a few seconds and deformations are limited to a bending in the middle body region (Pl. III 8, b).

The fine structure of the contractile apparatus seems to be sufficiently preserved in the presence of OsO_4 (the fixative 2), and addition of the acrolein (the fixative 3) may contribute to its better visualization (Pl. IV 10). In some regions of amoeba well fixed filaments are seen in threads running in various orientations in the neighbourhood of the cell membrane (Pl. IV 10, a), and in others the filaments show a very close contact with the outer membrane and the arrangement parallel to the cell surface (Pl. IV 10, b). According to many earlier findings (Grębecka and Hrebenda 1979, Wehland et al. 1979, Grębecki 1982 a, Stockem et al. 1982, 1983) the first situation is more common along the middle-posterior, and the second along the anterio-lateral body walls.

Discussion

Almost immediately after the introduction of the glutaraldehyde fixation to the purely morphological research (Sabatini et al. 1963, Fahimi and Drochmans 1965 a, b, Tokuyasu and Scherbaum 1965) as superior to the classical OsO_4 fixatives, Wohlfarth-Bottermann and Komnick (1966) warned against its uncontrolled use in the study of ultrastructural differentiations related to the cell motility. They demonstrated on *Amoeba proteus*, *Physarum polycephalum* and some other cells that the glutaraldehyde fixation is very slow, promotes contraction, intensifies the filaments production, and thus the apparently better image of the motile apparatus may be due to "intravitale oder supravitale Artifizierung". Our results fully confirmed these objections. Moreover, the cinematographic analysis revealed that the "lokale Cytoplasmakontraktionen" described by Wohlfarth-Bottermann and Komnick (1966) are not random, but have a well defined be-

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havioural expression in the regular sequence of streaming reversal and re-reversal phases. It should be emphasized again that this response was displayed, without any exception, by all specimens recorded by the time lapse filming.

We conclude that the streaming reversal in the glutaraldehyde + polyethylene glycol solution is a true motor response, and that this fixative acts, before stabilizing the motor apparatus, as a potent contracting agent. At the first step, it induces contraction of all advancing fronts; the effect is so powerful that initially it squeezes the endoplasm back, against the usual contractile activity of other cell regions. It seems that after 30—50 s the proper fixation is beginning, starting from the anterior cell regions, whereas the posterior ones are still capable to contract for 1—2.5 min longer (such gradiental action of drugs on amoebae is known from older literature — Hyman 1917). So, the former fronts gradually cease to exert pressure on the endoplasm, and the streaming may be reoriented again forwards. But it fails to restore the original shape of frontal pseudopodia, because their structure is then already stabilized by fixation in the contracting state. Finally, the gradual cessation of contraction in the posterior regions, due to their fixation, closes the cycle.

If this interpretation is correct, it should be recognized that this kind of fixation cannot reveal the subtle concurrence of peripheral contraction and frontal relaxation in amoeboid movement, because it demonstrates the whole motile apparatus in the contracting state, independently of the functional state it had before fixation in different cell regions. This interpretation may be reinforced by the fact, also mentioned by Stockem et al. (1982), that the addition of phalloidin to the glutaraldehyde fixative still "improves" the results. Probably the phalloidin may be helpful to protect actin *in vitro* (Gicquaud et al. 1980), but in a living amoeba it provokes very strong over-natural contraction of the whole cell cortex (Stockem et al. 1978, Taylor et al. 1980 a, b, Hoffmann et al. 1984), and thus it can only increase the artifactual influence of the fixative.

The physiological artifacts created by a too slow fixative which is simultaneously a contracting agent are easily produced, and particularly misleading in the interpretation of the mechanism of movement, at the both opposite poles of locomoting amoebae. So for example, the doubts expressed by Stockem et al. (1982) about the state of the cortical complex in the advancing pseudopodial tips might result from the fact that, in the glutaraldehyde + polyethylene glycol solution, each advancing pseudopodium becomes invariably contracted during fixation. The inexplicable abundance of hayline blisters in the uroidal region, reported in the same paper, probably represents another physiological artifact created by the motor response of amoeba to the fixative (produced when the tail region is swollen by the reversed endoplasmic stream).

The fixatives containing OsO_4 are safe from producing this type of physiological artifacts. They operate so fast that the contractile apparatus has no time to react and to produce any new motor response before fixation. Already 35 years ago an OsO_4 + $HgCl_2$ mixture allowed Párducz (1952) to fix correctly and study stage-by-stage even a so fast movement as the ciliary beating (see Párducz

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1967 for the review). The same fixative is still successfully applied to study the contractile system of slime moulds plasmodia working under various conditions (e.g. Wohlfarth-Bottermann et al. 1983). But on the other hand, with osmium, we run a risk of partly slackening the F-actin network (Maupin-Szamier and Pollard 1978, Gicquaud et al. 1980). Therefore, we can accept without reserve only such information about the functional differentiations of the cortical contractile layer of *Amoeba proteus* which is concurrently provided by application of different techniques to the fixed and living material.

We can firmly rely on data coming from different sources which consistently demonstrate that the motive force of amoeboid movement is generated along the whole cell periphery, whereas the structure of fronts makes them the most relaxed, weakest regions responsible for the control and steering of locomotion (according to the generalized cortical contraction theory of amoeboid movement — Grębecki 1982 a, b).

The presence of the contractile, and actually contracting, layer along the whole cell length has been demonstrated in the material fixed for EM by different methods (e.g. Rinaldi and Hrebenda 1975, Hauser 1978, Grębecka and Hrebenda 1979, Wehland et al. 1979, Stockem et al. 1982) in the glycerinated models of amoebae (Rinaldi et al. 1975), by immunocytochemical study of amoebae fixed at −10°C in methanol (Stockem et al. 1983 b), by fluorescence techniques applied to living cells (Gawlitta et al. 1980, Stockem et al. 1983 a) and by exposing different cell regions to strictly localized, contracting or relaxing photic stimuli (Grębecki 1981).

The specific structure of the advancing pseudopodial tips, in which the contractile cortex is absent beneath the outer membrane, but it forms deeper a plasmagel sheet separating the frontal hyaloplasm from the granuloplasm (through which the hyaloplasm may freely penetrate), was not only seen in the fixed material (Grębecka and Hrebenda 1979, Wehland et al. 1979), but also in living cells to which the fluorescein-labelled actin was microinjected (Gawlitta et al. 1980, Taylor et al. 1980 b, Stockem et al. 1983 a). Moreover, the sieve properties of the cortex detached from the membrane are confirmed in amoebae treated with phalloidin and some other drugs (Stockem et al. 1978, Taylor et al. 1980 a, b, Hoffmann et al. 1984). And last but not the least, such a structure of the front accords well with the plasmagel sheet role and behaviour deduced earlier from light microscope observation of frontal tips of living amoebae (Mast 1926, Rinaldi 1964 a, b).

It should be concluded in general that the results provided by glutaraldehyde fixation become conclusive only after being directly or indirectly confirmed by other methods, preferably *in vivo*.

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EXPLANATION OF PLATES I–IV

1: Two examples of the size, shape and position changes of amoebae during fixation in the glutaraldehyde + polyethylene glycol mixture. Redrawn from 1 frame/s cinematographic records. Black contour – the last frame before adding the fixative, white profile – the final fixation stage
2: Cinematographic analysis of the size, shape and position changes of two amoebae before fixation, during the glutaraldehyde-induced reversal of endoplasmic flow, and from the moment of streaming renormalization up to the end of the fixation procedure. At each stage the black contour represents the initial and the white profile the final outline of amoeba body. Arrows indicate the direction of the endoplasmic stream. The same scale as in 1.
3: Polytactic amoeba about 1 s before adding the glutaraldehyde and polyethylene glycol solution (a), 15 s later at the streaming reversal stage (b), and 1.5 min later during the streaming renormalization period preceding the final fixation (c). In 3–6 and 9 arrows indicate the endoplasm flow direction, asterisks the fixed stationary state, and the bars equal to 150 μm
4: Frontal pseudopodia just before adding the glutaraldehyde with polyethylene glycol (a), and in the fixed state 2.5 min later (b)
5: Normal shape of the tail region of a polytactic amoeba about 1 s before adding the same fixative (a) and its dilatation in the fixed state 3 min later (b)
6: Monotactic amoeba just before contact with the fixative (a), in the 25th s of exposal to the glutaraldehyde and polyethylene glycol at the streaming reversal phase (b), and 3 min later in the fixed state (c)
7: Deformations of the vesicular frontal cap of monotactic amoebae by the glutaraldehyde + polyethylene glycol fixative. Constriction at the cap basis (a), its flattening (b), and the full collapse (c)
8: Cinematographic analysis of the fixation of two polytactic amoebae in the glutaraldehyde + + OsO_4 mixture, shown in the same way as in 2. Asterisks symbolize the extinction of any movements
9: Polytactic amoeba about 1 s before fixation (a), and in the fixed state 2–3 s later (b), after treating with the glutaraldehyde + OsO_4 solution
10: The filamentous layer (FL) in the body walls of *A. proteus*, demonstrating in some regions a less regular arrangement (a) and in others a close contact of parallel actin filaments with the cell membrane (b). Connections between the membrane and filaments are indicated by small arrows. Bars – 0.5 μm

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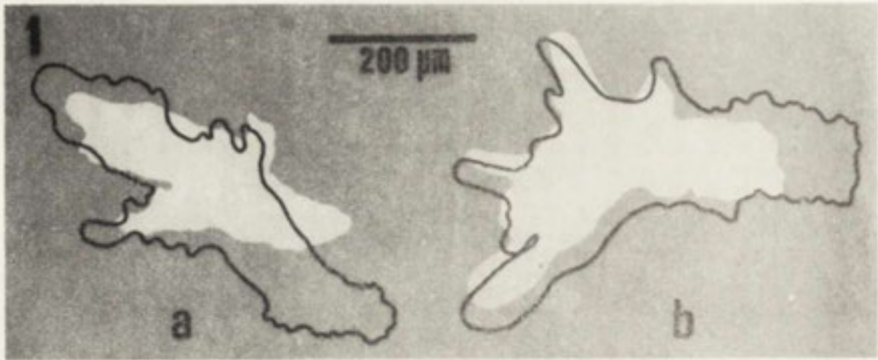

2
a
0–54"
normal movement
fixation
reverse streaming
streaming re-normalized
b
0–48"
48–96"

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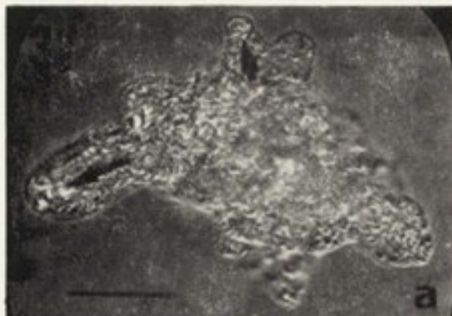

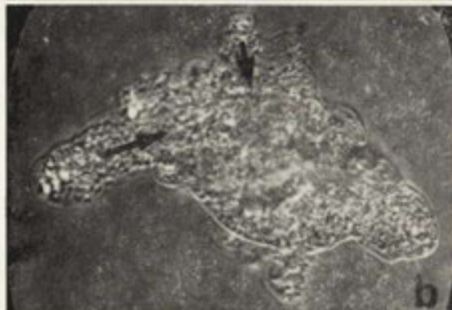

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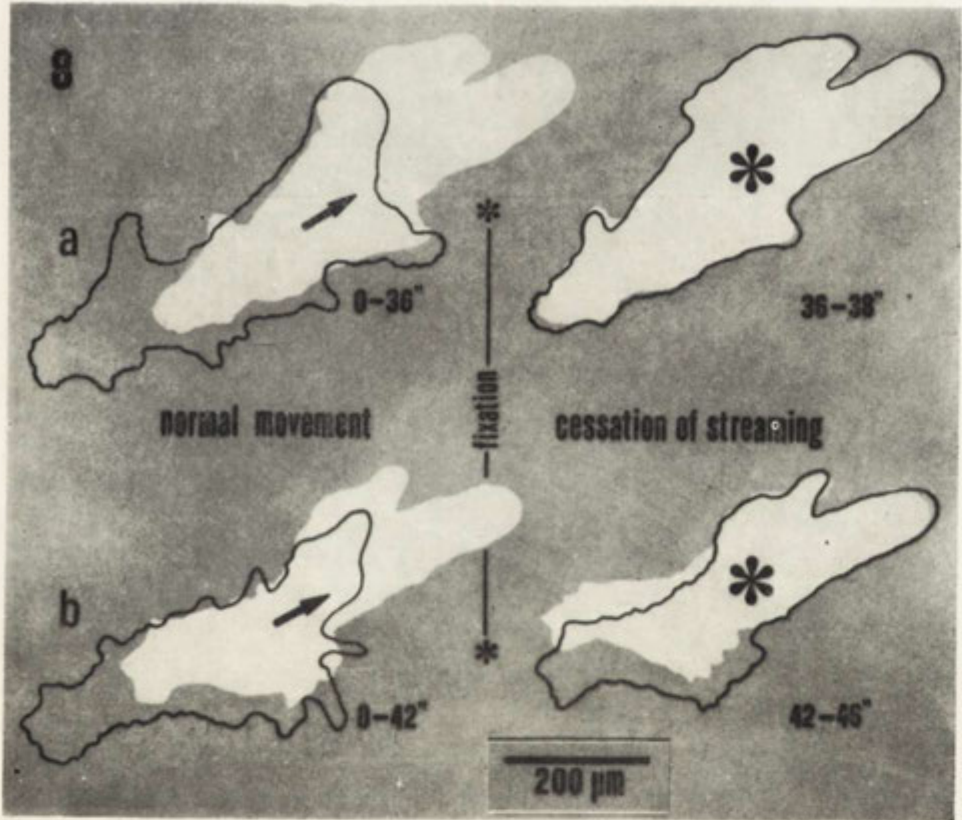

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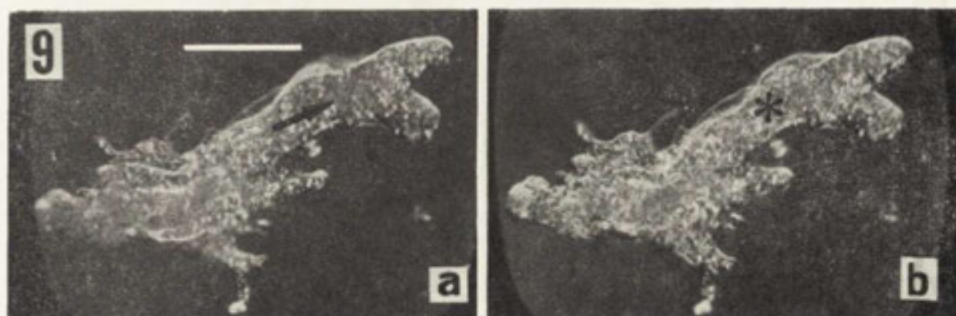

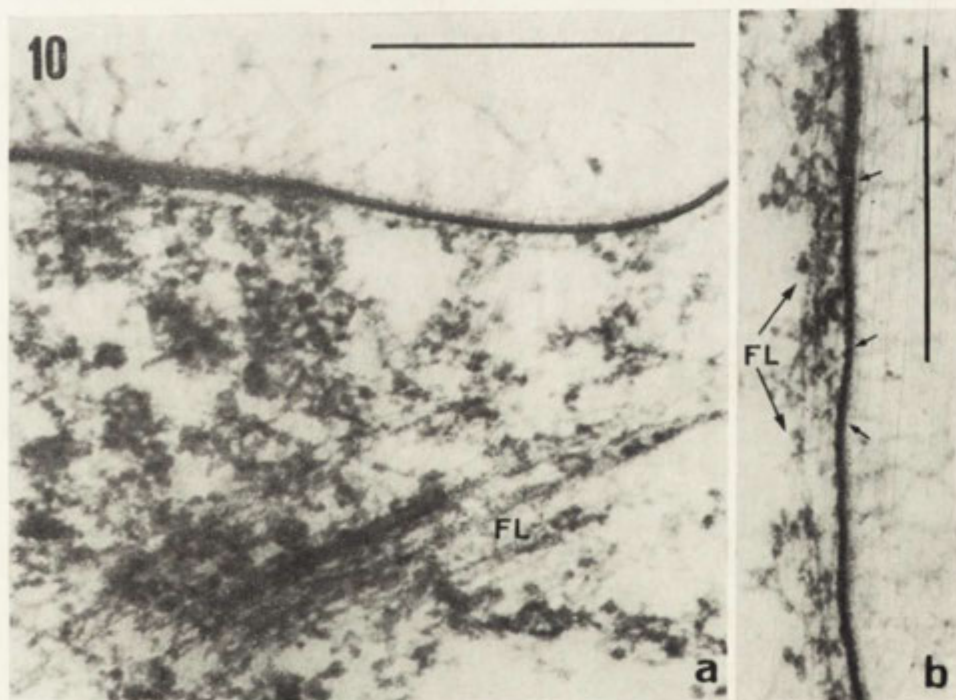

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The Influence of Arginine Vasopressin (AVP) on Phagocytosis in the Unicellular *Tetrahymena*

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Synopsis. Arginine-vasopressin (AVP) depressed the phagocytotic activity of the *Tetrahymena* in the concentration range $10^{-7}-10^{-11}$ M. The effect of primary interaction was durable. The depressive effect of $10^{-8}-10^{-9}$ M AVP, and the significant phagocytosis stimulant effect of 10^{-10} M AVP persisted in the progeny generations without reexposure to AVP. The second interaction caused no further depression or stimulation at any tested concentration except 10^{-9} M, which accounted for a further significant stimulation of phagocytosis in the cultures preexposed to 10^{-9} or 10^{-10} M AVP. It follows that organisms at the lowest levels of phylogenesis are also able to respond to the vertebrate hormone vasopressin and to develop a "memory" of interaction.

The hormones of higher (vertebrate) organisms also act on invertebrates, even on unicellulars (Csaba 1980, 1981). For example the unicellular *Tetrahymena* displays membrane patterns which are able to bind vertebrate hormones by a receptor-like mechanism, and since it also comprises an adenyl-cyclase cyclic AMP system (Kariya et al. 1974, Kassis and Kindler 1975, Kuno et al. 1981, Csaba and Sudár 1985) the information received by hormone binding is mediated intracellulary and evokes a specific (vertebrate-like) or non-specific (metabolic) response (Csaba 1985). Serotonin and histamine have been shown to stimulate the phagocytosis of the *Tetrahymena* (Csaba and Lantos 1973), thyroxine and thyroxine precursors its mitotic activity (Csaba and Németh 1975), whereas insulin and adrenalin alter its glucose metabolism (Csaba and Lantos 1975). Other polypeptide hormones, to which the *Tetrahymena* does not present a "target organ", as e.g., TSH, FSH and ACTH, act on its RNA synthesis (Csaba and Ubornyák 1982). Primary interaction with the hormone gives rise to hormonal imprinting, which accounts for alteration of a certain cellular function (Csaba 1985, Csaba et al. 1985), more precisely, of cellular response, to judge from the latter's relative change (usally increase) on reexposure (Csaba 1985).

In the present study we tested arginine-vasopressin (AVP) for influence on the phagocytosis of the *Tetrahymena* as an index of its response. Vasopressin, being

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an oligopeptide comprised of nine amino acids, differs radically from all amino-acid or polypeptide hormones previously tested for effect on the unicellular. It therefore seemed worthwhile to examine it also for imprinting potential in the given experimental model.

Tetrahymena pyriformis GL cells, cultured for two days in 0.1 per cent yeast extract containing 1 per cent Bacto tryptone medium (Difco, Michigan, USA) for two days at 28°C were used in the logarithmic phase of growth. Part of the mass cultures was not treated to serve as control, part was exposed to 10^{-8}, 10^{-9}, or 10^{-10} M AVP for 24 h, centrifuged, washed and transferred to physiological Losina salt solution for 24 h. Then 0.5 ml samples of each culture were combined with 0.5 ml Chinese ink suspension and 0.5 ml AVP (range: $10^{-7}-10^{-11}$ M, in Losina solution). The control cultures not pretreated with AVP were incubated in presence of AVP + Chinese ink for 30 min, during which they were sampled at 5, 10 15, 20 and 30 min, whereas those pretreated with AVP were sampled at 10 and 20 min of reexposure. At each sampling interval, phagocytosis was arrested by addition of 0.5 ml 4 per cent formaline solution (in PBS), the cells were centrifuged, the sediment was resuspended in 0.5 ml Losina solution, spread on slides and dried for counting the Chinese ink containing vacuoles in 50 cells per slide. Each experiment was performed in five replicates, from which were derived the mean values shown in Table 1 and Fig. 1. Significances had been evaluated by Student's "*t*" test.

Table 1

Vacuole counts in *Tetrahymena* cells treated with different concentrations of AVP with or without preexposure to it at various levels

AVP concentration (second exposure)	Untreated control		Primary exposure to various concentration of AVP					
			10^{-8} M		10^{-9} M		10^{-10} M	
	exposure time in min		exposure time in min					
	10	20	10	20	10	20	10	20
0	3.6	5.93	2.71[c]	4.72[c]	3.33	5.11	4.42[c]	7.04[c]
10^{-7} M	2.84[a]	5.36	2.45	4.64	3.34	5.33	4.1[c]	7.22[c]
10^{-8} M	2.96[a]	5.62	2.76	4.82[c]	3.09	5.13[c]	4.32[c]	7.18[c]
10^{-9} M	3.59	5.01[a]	2.65[c]	4.94	3.69[b]	5.91[b,c]	4.66[c]	7.52[b,c]
10^{-10} M	3.6	5.2[a]	2.63[c]	4.86	3.44	5.42	4.53[c]	6.98[c]
10^{-11} M	3.33[a]	5.75	2.63[c]	4.76[c]	3.25	5.38	4.24[c]	6.96[c]

[a] – significance of difference between once exposed and unexposed cells ($p < 0.05$), [b] – significance of difference related to only "primary exposure" values in the same column ($p < 0.05$), [c] – significance of difference from untreated cells (values in first and second column) ($p < 0.05$; $p < 0.01$)

The results of a single exposure to AVP are shown in Fig. 1. The vacuole counts did not differ between the treated and untreated cells at 5 min, but showed a significant ($p < 0.01$ or $p < 0.05$) decrease relative to the control at three levels of

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AVP (10^{-7}, 10^{-8}, 10^{-11} M) by 10 min. The differences were not significant at 15 min, and the vacuole counts of all treated cells were lower than the control at 20 min significantly in the cultures exposed to 10^{-9} and 10^{-10} M. At 30 min all treated cultures still showed lower vacuole counts than the control, under the same relations of significance as at 10 min.

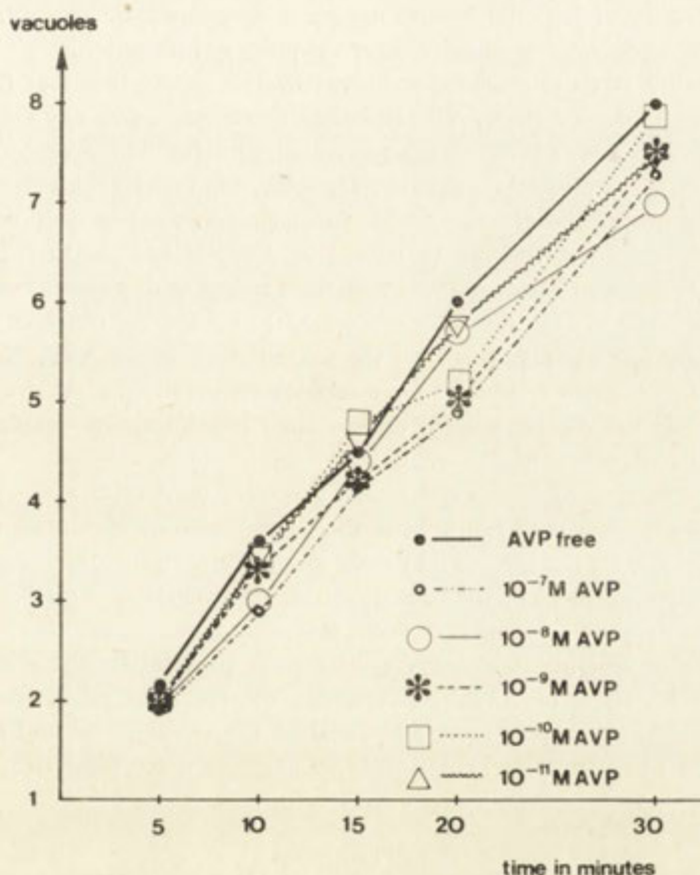

Fig. 1. Influence of different concentrations of AVP to the phagocytotic (vacuole count) of *Tetrahymena* (significances of the different points in the text)

It follows that the *Tetrahymena* was not indifferent to treatment with AVP. The significance of relative vacuole count decreases at the 10^{-10} and 10^{-11} M levels of AVP treatment signified the non-toxic nature of the effect.

As shown in Table 1, the phagocytosis depressing action of 10^{-8} M AVP was durable, having been still demonstrable after 24 h; the extent of phagocytotic activity was significantly lower without reexposure. 10^{-9} M AVP also accounted for depression at both 10 and 20 min although to a not significant degree. However, at 10^{-10} M, the phagocytosis depressant action became reversed, to judge from a significant increase in phagocytic activity without reexposure, at 24 h.

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As already noted in the introduction, induction of durable functional alterations by a hormone has been repeatedly observed (Csaba 1985, Csaba et al. 1985). The concentration-dependence of that effect in the case of vasopressin is nevertheless remarkable: while at the higher (from the point of view of the receptor still physiological) concentrations of vasopressin (10^{-8}, 10^{-9} M), the change induced by the primary interaction persisted with or without reexposure, at its lower concentrations the functional change (increase in phagocytic activity) appeared on second occasion without precedence at the primary interaction.

The stability of these effects is indicated by the observation that the cultures pretreated with 10^{-8} or 10^{-9} AVP responded to reexposure at any concentration by a relative decrease, whereas those pretreated with 10^{-10} M AVP by significant relative increase, in phagocytic activity. However, the latter values increased only exceptionally (on reexposure at 10^{-9} M) to a significant extent over those shown by cultures preexposed, but not reexposed to AVP. It appears that, in the case of AVP, imprinting does not follow the pattern common to most hormones, inasmuch as the usual change (increase or decrease) in response, observed at reexposure, generally did not take place on the second exposure to AVP. Nevertheless, the significant differences observed on reexposure to 10^{-9} M AVP support the hypothesis that the regular pattern of hormonal imprinting also applies to AVP within a limited concentration range.

The present and former pertinent experimental results (Csaba et al. 1982) unequivocally support the hypothesis of the transmission of imprinting to the progeny generations, on the ground that since seven to eight generation changes occur in the *Tetrahymena* in 24 h, the cells involved in reexposure represented a relatively distant progeny generation of those preexposed.

The present experimental observations cannot substantiate the implication of a receptor-level effect of AVP. Nevertheless, the fact that AVP, which acts in higher organism at receptor level, also acted on the *Tetrahymena*, and most intensively at the lower concentrations, strongly suggests a receptor-level action.

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Comparative Investigations on Metabolism of Pathogenic and Non-pathogenic Strains of Free-living Amoeba[1]

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Synopsis. A comparative study of pathogenic and non-pathogenic strains of *Naegleria* and *Acanthamoeba* performed in late logarithmic phase of growth reveals a higher enzymatic and bioenergetic activity in the pathogenic *Acanthamoeba* strain. Cytochrome oxidase activity was much higher in both *Acanthamoeba* and *Naegleria* pathogenic strains, cyanide sensitivity being similar in both strains. The presence of special rotenone-insensitive dehydrogenase oxidizing exogenous NADH in both *Naegleria* strains could not be excluded. A high activity of decarboxylating malate dehydrogenase and its regulatory properties in the pathogenic strains only may indicate a better adaptive abilities of those pathogenic strains.

Reports of free-living amoeba infection involving human brain, skin, lung and eye have increased markedly during the last decade. Pathogenicity of certain strains of *Acanthamoeba* and *Naegleria* is well known (Kasprzak et al. 1982, Martinez 1983, Kasprzak 1985). The amphizoic strains of *Acanthamoeba* and *Naegleria* live in well oxygenated natural environment including soil, fresh water and sea water (Marc-Daggett et al. 1983). The pathogenic strains of both genera live in human tissues with a considerable amount of oxygen (lung — Kasprzak et al. 1974, Kasprzak 1985, brain — Wong et al. 1976) and *Naegleria fowleri* strains do not even reproduce in anaerobic conditions (Weik and John 1977).

Considerations on the metabolism of the amphizoic amoeba strains and in particular their bioenergetic metabolism and adaptation abilities of the key enzymes of their metabolic pathways seem to be very interesting from the theoretical point of view.

Bearing in mind that the metabolism of both free-living and pathogenic amoeba of the two genera mentioned above should be adapted to the relatively well oxygenated environment it seems to be logical that the ultrastructural model of their ameboid stages shows the presence of mitochondria with a high condensed matrix

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as an example of common characteristic. Basing on their electron microscopic studies, Visvesvara and Callaway (1974) and Carosi et al. (1977) concluded that in *N. fowleri* both dumbbell-shaped and cylindrical and oval mitochondria could be found. According to his results Lastovica (1975) stated that the greater surface area offered by cup-shaped or dumbbell-shaped mitochondria observed in pathogenic *Naegleria* strains may be required for their increased metabolic activity.

The bioenergetic metabolism of non-pathogenic *Acanthamoeba* strain Neff was investigated intensively in the past decade (Edwards and Lloyd 1978, Lloyd and Griffiths 1968 and Hryniewiecka et al. 1978, Hryniewiecka 1980, 1985), but the bioenergetic metabolism of pathogenic *Naegleria* strain (Lee) was studied by Weik and John (1979) only. The latter authors found that this metabolism seems to show many features characteristic of mammalian mitochondria. According to Edwards and Lloyd (1978) and Hryniewiecka (1980), in the Neff strain of *Acanthamoeba* there is a coexistence of normal electron transport chain and the alternative one, nonphosphorylating and insensitive to cyanide; the ratios in electron flow trough a branched electron transport chain and sensitivity to cyanide being different in different growth stages of the amoeba. Hryniewiecka (1980) found three sites of phosphorylation with substrates oxidized *via* NAD and two sites with succinate in classical electron transport chain in mitochondria of the Neff strain of *A. castellanii*. Her recent results (1985) showed in the Neff strain of *A. castellanii* a striking similarity to the characteristics of malate oxidation in plant mitochondria. On the other hand, Hryniewiecka questions Rustin's et al. (1980) hypothesis that the electrons transferred from malate by decarboxylating malate dehydrogenase is directed to the alternative oxidase only and the electrons transferred by malate dehydrogenase to the classical cytochrome chain.

The comparative biochemistry of pathogenic and non-pathogenic *Acanthamoeba* and *Naegleria* strains has so far been largely neglected and the following study sets out as an initial objective an examination of their bioenergetic metabolism and the regulatory properties of some carbohydrate metabolism enzymes.

Some results of those investigations were already presented (Boczoń et al. 1978).

Material and Methods

Cultivation of amoeba strains. After some preliminary examination of the effect of aeration on the growth period of pathogenic amoeba strains, the following two pairs of strains, pathogenic vs. non-pathogenic, of the same species were selected for our biochemical studies: *Acanthamoeba castellanii* strain 309 (pathogenic for mice isolated from the environment by Kasprzak and Mazur 1974) and strain Neff (non-pathogenic, isolated by Neff 1957) and *Naegleria fowleri* strain Vitek (pathogenic for mice, isolated from a patient with PAME by Cerva 1969) and strain 5D (non-pathogenic, isolated by Kasprzak and Mazur 1978).

A cultivation of *Acanthamoeba* strains (Neff and 309) was performed according to the test described by Mazur et al. (1982). Using the Bactocasitone medium + 10% horse serum, the maximum growth was reached between the 3rd and 4th day of culture. The *Naegleria* strains (5D and Vitek) were grown on Bactocasitone + 10% sheep serum, which can provide similar growth con-

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ditions for the amoeba as the recommended Calyg medium (Cerva 1977), but the maximum of growth was reached as early as on the 4th day. Since the late logarithmic phase of growth (4th day) was to be investigated, the Bactocasitone + sheep serum medium was more useful for our estimations. Both the *Acanthamoeba* strains were grown in axenic culture without aeration at 24°C and both the *Naegleria* strains at 37°C.

In the late logarithmic phase of growth (after 96 h of culture), or in some experiments in the stationary phase of growth (144 h) the amoeba were harvested by centrifugation at room temperature at 800 g for 4 min followed by 3-times washing in water.

Enzymatic assays. For enzymatic investigations two types of homogenates (20%w/v) were prepared:

(1) in ionic medium — (I) containing 5% potassium sulphate, 0.5% betamercaptoethanol and 50 mM Tris-glycine buffer (pH 8.5)

(2) in sucrose medium — (S) containing 250 mM sucrose and 20 mM Tris buffer (pH 7.5)

The enzymatic activity of the following enzymes was measured (at 24°C): malate dehydrogenase — MDH (EC 1.1.1.37), NAD- and NADP-specific decarboxylating malate dehydrogenase (EC 1.1.1.39 and EC 1.1.1.40) and phosphoenolpyruvate carboxykinase — PEPCK (EC 1.1.1.32) according to Körting and Barrett method (1977), succinate dehydrogenase — SDH (EC 1.3.99.1) following the King's method (1963), NADH-linked fumarate reductase (FR) and the NADH oxidase employing the Prichard's method (1973) and lactate dehydrogenase — LDH (EC 1.1.1.27) by King's method (1965). Components of the incubation media were as follows:

Malate dehydrogenase — 100 mM phosphate buffer, pH 7.5, 3 mM oxaloacetate, 0.2 mM NADH

Lactate dehydrogenase — like in MDH except 3 mM pyruvate was used as a substrate

NAD(H)-specific decarboxylating malate dehydrogenase — 60 mM Tris-HCl buffer, pH 8.0, 8 or 10 mM malate (depending on the strain), 2 mM $MnCl_2$, 0.4 mM NAD(H)

PEPCK — 150 mM Tris-HCl buffer, pH 6.0 or 7.0 (depending on the strain), 5 mM $MnCl_2$, 20 mM $NaHCO_3$, 2 mM IDP, 0.5 mM NADH, 2.5 units of MDH and 15 mM phosphoenolpyruvate

NADH-linked FR — 400 mM phosphate buffer, pH 6.9, 33 μM $CaCl_2$, 0.1 mM NADH and 10 mM fumarate

Succinate dehydrogenase — 50 mM phosphate buffer, pH 7.6—7.8, 20 mM succinate, 0.1% of BSA, 1 mM KCN, 0.05 mM DCIP and 1 mM PMS

The yield was 2—3 mg of mitochondrial protein (*Acanthamoeba* strains) and 4—6 of mitochondrial protein (*Naegleria* strains) per 1 g of fresh weight of amoeba.

Oxygraphic measurements. The homogenates used in oxygraphic measurements were prepared in sucrose medium with the following components added: 1 mM EDTA and 0.2—0.5% of BSA (fatty acid free, fraction V, Sigma). Mitochondria were isolated from sucrose homogenates by differential centrifugation (1st spinning at 1000 g for 6 min, 2nd spinning of the 1st supernatant at 10 000 g for 15 min). Mitochondrial pellet obtained after the 2nd spinning was washed twice prior to the final resuspension in sucrose medium without BSA and EDTA. Oxygen consumption was measured amperometrically by a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C in incubation medium containing: 300 mM sucrose, 10 mM Tris buffer, pH 7.2, 6 mM $MgCl_2$, 10 mM phosphate buffer, 0.2 mM EDTA, 10 mM KCl, 0.2% of BSA (FAF, Sigma), 10 mM succinate, malate and alpha-ketoglutarate, 1 mM NADH and 260—300 μM ADP.

Respiratory control index (RCI) was calculated according to Estabrook (1967). The following inhibitors were added in 2—10 μl volumes: sodium cyanide — KCN (aqueous solution) — 100—1000 μM, salicylhydroxamic acid — SHAM (aqueous solution) — 300 μM, rotenone (in formamide) — 0.2 mg/ml and antimycine A (in ethanol solution) — 500 μg/ml.

Protein was estimated by the Lowry et al. method (1951).

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Results

A sufficient quantity of the investigated strains was difficult to obtain and therefore the number of experiments on mitochondrial fractions obtained after differential centrifugation of homogenates was small.

Table 1 shows the respiratory rate (QO_2) in State 4 with different substrates of mitochondrial fractions isolated from pathogenic and non-pathogenic amoeba

Table 1

Rates of respiration in State 4 (QO_2) of mitochondria isolated from pathogenic (309 and Vitek) and non-pathogenic (Neff and 5D) amoeba strains

Substrate	*Acanthamoeba castellanii*		*Naegleria fowleri*	
	Neff	309	5D	Vitek
Ascorbate/TMPD	38 30–45	117 113–120	37 35–41	114 110–117
NADH	33 31–34	30 29–33	17* ±12	95 90–100
Succinate	17 15–19	15 14–16	8* ±4	26* ±10
Malate	3 2–4	2 2–3	— —	15 —
Malate + NAD	12°	16°	—	—
Alpha-ketoglutarate	2 1–3	3 2–4	1 0–2	9 7–11
Endogenous	2 1–4	6 3–7	10 8–12	9 3–9

The oxygen consumption rate (QO_2) corrected for endogenous respiration was expressed in nA O_2/min/mg of mitochondrial protein. Mitochondrial preparation (about 2 mg of protein per vial) was incubated at 30°C in 1 ml of the following medium: 300 mM sucrose, 10 mM Tris buffer (pH 7.2), $MgCl_2$ – 6 mM, 0.2% BSA, 0.2mM EDTA, 10 mM KCl, 0.2 mM TMPD, 4 mM ascorbate, 10 mM substrates (20 mM succinate only). Values are the mean of 3 experiments (* – the mean of 6 experiments, ° – the mean from 2 experiments)

strains. The endogenous respiration was low in the Neff strain (1–4 nA O_2/min/mg of protein) and highest in strain 5D (8–12 nA O_2/min/mg of protein). The high respiratory rate with NADH reaches the highest value in *Naegleria* pathogenic strain Vitek (6 times higher than in non-pathogenic strain 5D). State 4 respiration was stimulated by the following substrates in a decreasing order: succinate, malate and alpha-keto-glutarate. In two preliminary experiments addition of NAD increased considerably the respiration with malate.

The most striking difference was observed in pathogenic and non-pathogenic strains in respiration rate with ascorbate /TMPD couple, 3 times higher rate was observed in pathogenic than in non-pathogenic strains of both species.

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The influence of SHAM — a specific inhibitor of an alternative pathway — was checked in both non-pathogenic strains: Neff and 5D only. Respiration with ascorbate/TMPD couple was sensitive to much lower SHAM concentration in the Neff strain than in the 5D strain (almost total inhibition at 250 μM and 2000 μM concentration, respectively, when both inhibitors (KCN + SHAM) were added the inhibitory effect in strain 5D was additive. Evidently more data on both pathogenic and non-pathogenic strains are needed to evaluate the problem of the presence of alternative pathways in electron transport chains of those pathogenic strains.

The effect of some inhibitors on State 4 respiration with NADH, ascorbate/TMPD couple and succinate in total homogenates of amoeba strains is presented in Table 2. The respiration with ascorbate/TMPD couple and succinate was largely inhibitited by about 1 mM cyanide, but no significant difference in this sensitivity to inhibitors was found between pathogenic and non-pathogenic strains. The oxidation of succinate, malate and NADH was completely inhibited in strain Neff by 1500 μg/mg of protein antimycin (data not presented in Table 2).

Table 2

Effect of some inhibitors on the State 4 respiration in mitochondria of pathogenic (309 and Vítek) and non-pathogenic (Neff and 5D) amoeba strains

Strain	Inhibitor	Ascorbate/TMPD	NADH	Succinate
Neff	KCN	97 (700)	—	95 (850)
	SHAM	100 (250)	—	—
	Rotenone	—	0 (30)	
309	KCN	88 (1200)	—	—
5D	KCN	73 (1000)	—	85 (680)
	SHAM	70 (1500)	—	—
	SHAM + KCN	100 (1500+1000)	—	100 (1000+500)
	Rotenone	—	56 (20)	—
Vítek	KCN	100 (840)	—	100 (750)
	Rotenone	—	40 (30)	—

Inhibition of respiration by cyanide, SHAM or rotenone expressed in %. Concentrations of the inhibitors (in μm) in parantheses next to the value. All values are the mean of 3 experiments. Respiratory activity with ascorbate/TMPD couple, succinate and NADH with no inhibitor were as shown in Table 1.

Only a partial sensitivity to rotenone of the exogenous NADH oxidation (about 50% inhibition by about 25 μM rotenone) was observed in both strains of *Naegleria* and a total insensitivity in the non-pathogenic strain of *Acanthamoeba* (Neff).

The oxygen uptake was not increased when up to 500 μM ADP was added; the only exception was the respiratory control index 1.2—1.5 without clear State 4 to State 3 transition measured with succinate in Neff and Vítek strain mitochondria.

The activity of the following enzymes: LDH, MDH, SDH, FR and both de-

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carboxylating malate dehydrogenases, which may be involved in modified glycolytic pathway was measured parallel in two extraction media, but the highest values obtained in a medium of choice for individual enzymes are presented in Table 3.

Table 3
Activity of some enzymes of the modified glycolytic pathway in total homogenates of amoeba in late phase of logarithmic growth (4th day)

Enzymes	*Acanthamoeba castellanii*		*Naegleria fowleri*	
	Neff	309	5D	Vitek
PEPCK	19 16–21	29 25–33	22 20–24	29 25–33
MDH	1930 1830–2000	1742 1000–2484	2103* ±630	2138 2000–2276
LDH	33 30–35	54 50–59	45 40–50	11 8–13
NAD-specific decarboxylating malate dehydrogenase	114 90–138	168 120–216	339* ±101	243 240–246
NAD-specific decarboxylating malate dehydrogenase	44 40–58	38 20–46	71 60–83	23* ±8
SDH	2 1–3	7 5–9	15 11–19	8 5–11
NADH-FR	3 2–5	9 5–13	0 0–1	4 2–6
NADH-OX	16 13–18	16 10–20	8 4–12	– –

Activity (expressed in nmoles/min/mg of protein) was measured in sucrose medium (see Material and Methods) after thawing and freezing 3-times, except the activity of MDH and both decarboxylating malate dehydrogenases where the ionic medium was chosen for better extraction. Results are the mean of 3 experiments (*6 experiments, the mean±SD). LDH – lactate dehydrogenase, MDH – malate dehydrogenase, SDH – succinate dehydrogenase, NADH-FR – fumarate reductase, PEPCK – phosphoenolpyruvate carboxykinase, NADH-OX – NADH-oxidase.

The established pH and substrate concentration optima are as follows:

MDH – pH 7.5 for all strains, substrate – narrow optimum about 1 mM in 309 strain, broad optimum (0.3–1.0) in both *Naegleria* strains

LDH – pH 7.5 for all strains, substrate – about 1 mM, higher (2.6 mM) in Vitek strain only

NAD-specific decarboxylating malate dehydrogenase – pH 8.0, substrate – about 6 mM for both non-pathogenic strains and about 16 mM for both pathogenic ones

NADP-specific decarboxylating malate dehydrogenase – pH broad optimum (8.4–9.0), substrate 6 Mm

PEPCK – pH 6.0 (in both *Acanthamoeba* strains) or 7.0 (in both *Naegleria* strains), substrate about 15 mM (checked in 309 and 5D only).

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The results of some experiments on the intracellular localization of all the enzymes tested (measured after differential centrifugation of sucrose, unfrozen homogenates), which are not presented in Table 3, show the exclusively mitochondrial localization of SDH and NADH-oxidase and localization in two compartments (mitochondrial and cytosolic) of MDH and both decarboxylating malate dehydrogenases; about 20—40% of total activities (depending on the strain tested) was found in the mitochondrial fractions.

Preliminary comparison of activity in the late logarithmic phase of growth (4th day) and in the stationary phase (6th day) reveals that during the aging of cultures the activity of LDH and SDH increases and that of decarboxylating malate dehydrogenase decreases.

General conclusions from the enzymatic estimations are as follows: (1) the activity of all enzymes tested except the MDH and NAD-specific decarboxylating malate dehydrogenase was many times lower than that in mammalian tissues, which made the procedure of measurements very difficult and in the case of FR almost impossible; (2) in *Acanthamoeba* strains the activity of most of the enzymes was 2—3-times higher in the pathogenic strain than in the non-pathogenic one. In *Naegleria* strains, however, the activity was similar an interesting exception being the LDH and NADP-specific decarboxylating malate dehydrogenase where the reverse situation was observed (the activity in non-pathogenic strain 5D was 3—4-times higher than in non-pathogenic strain Vítek).

The decarboxylating malate dehydrogenase was more thoroughly investigated, as it is a well known enzyme of potential regulatory properties in many organisms.

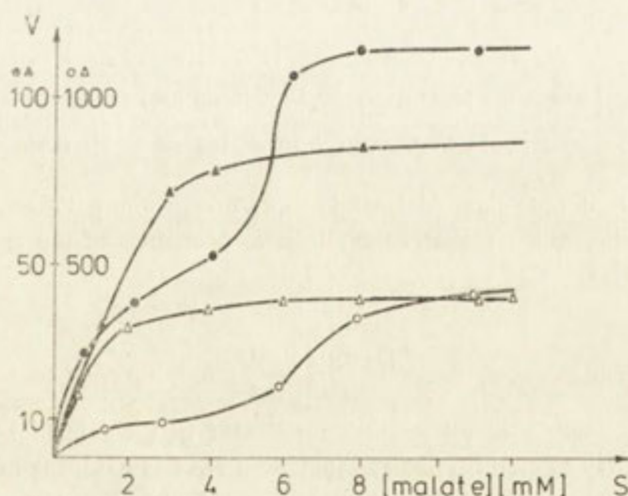

Fig. 1. Substrate saturation curves with malate for NAD-specific decarboxylating malate dehydrogenase in *Acanthamoeba* strains (filled circle — 309 strain, filled triangle — Neff strain) and *Naegleria* strains (open circle — Vítek strain, open triangle — 5D strain). Results are the mean of two experiments. The enzyme activities were in *N. fowleri* strains (Vítek and 5D) and *Acanthamoeba* strains (309 and Neff) as follows: 400, 380, 110 and 80 respectively. 0—100 V scale for strain 309 and Neff, 0—1000 V scale — for strain 5D and Vítek. The enzymes activity was determined as described in Material and Methods except for the concentration of malate stated on the abscissa

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The investigation of the influence of some effectors reveals a 100% enzyme inhibition by about 1 mM oxaloacetate (in pathogenic strain Vítek just 0.1 mM oxaloacetate was inhibitory). Fumarate — acting as a positive effector on regulatory enzymes from other sources — stimulated slightly the enzyme from the Vítek strain only (25% stimulation by 1.8 mM fumarate). Figure 2 presented the correlation between the activity of amoeba NAD-specific decarboxylating malate dehydrogenases and the concentration of Mg^{++} -ions.

The shape of substrate saturation curves was checked for NAD-specific decarboxylating malate dehydrogenases from all strains, as the sigmoidal shape may indicate possible allosteric properties of these enzymes. The substrate saturation curves are presented in Fig. 1. Those curves in both the pathogenic and non-patho-

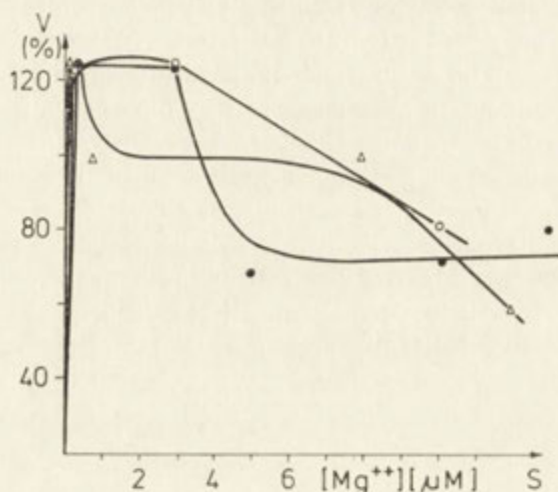

Fig. 2. Correlation between the Mg^{++}-ions concentration (in μM) and the NAD-specific decarboxylating malate dehydrogenases activity. The results are the mean of 3 experiments. Filled circle — 309 strain, open circle — Vítek strain, triangle — 5D strain

genic strains are double sigmoidal, while those of the non-pathogenic strains are typical hyperbolic, which suggests the allosteric properties of the enzymes in the pathogenic strains.

Discussion

Crude mitochondria of all strains investigated in the late logarithmic phase of growth (4th day of culture) had succinate and NADH oxidizing activity and relatively high ascorbate/TMPD couple oxidizing activity. In contrast to the other strains (*A. castellanii* Neff, Hryniewiecka 1980, and *N. fowleri*, Weik and John 1979) the alpha ketoglutarate and malate oxidizing activities in our studies were very low and the oxygen uptake supported by malate in both *Acanthamoeba* strains was strongly stimulated by exogenous NAD. Those mitochondrial preparations demonstrate, however, some common characteristics with B-type mitochondria

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from *A. castellanii* isolated by Lloyd and Griffiths (1968) and Edwards and Lloyd (1978) and the *A. castellanii* strain Neff prepared by Hryniewiecka (1980).

The high NADH-oxidation (especially in the Vítek strain) and the uncoupling of most mitochondrial preparations may indicate that they were partly damaged. Coupled mitochondria from the investigated amoeba strains were hard to obtain mainly due to the lack of sufficient amount of those strains (especially strain 5D and Vítek). The other possibility is that the oxidation of exogenous NADH might be their characteristic feature, which they share with the mitochondria of higher plants, as it was proved by Hryniewiecka for *A. castellanii* strain Neff (1985).

Despite the shortcomings of methodology, some general conclusions could be drawn from our results. There is a striking difference in ascorbate/TMPD couple oxidizing activity between pathogenic and non-pathogenic strains. Since the increase in this activity (by about 25%) with added cytochrome c may be interpreted as evidence of active cytochrome c oxidase, this observation is important and points out the higher cytochrome oxidase activity as a specific feature of pathogenic strains in both genera *Acanthamoeba* and *Naegleria*. The 3-fold increase in cytochrome oxidase activity in pathogenic strains was accompanied in the pathogenic strain Vítek by 3-fold increase in succinate oxidase activity.

Our preliminary inhibitors studies using crude mitochondrial preparations from all amoeba strains showed that the ascorbate/TMPD couple oxidation was sensitive to about 1 mM KCN inhibiting the cytochrome oxidase completely in mammalian mitochondria and partially in some protozoa, e.g. amoeba (*A. castellanii* Neff — B-type mitochondria, Edwards and Lloyd 1978) or different life cycle forms of *T. cruzi* (Rogerson and Gutteridge 1979). The only exception was the oxidase in strain 5D where just a combination of 1 mM KCN and about 1.5 mM SHAM caused a total inhibition, which may suggest the presence of branched chain electron transport system in this strain.

The total lack of sensitivity to the 30 μM rotenone of the NADH-oxidation in *A. castellanii* Neff, observed in our investigations, is consistent with Hryniewiecka's findings that the oxygen uptake with exogenous NADH in the same strain was insensitive up to 50 μM rotenone. Only partial sensitivity of the NADH-oxidation to rotenone (about 45% by 25 μM rotenone, see Table 2) in both *Naegleria* strains may indicate the presence of special dehydrogenase insensitive to rotenone and oxidizing the exogenous NADH.

The response to inhibitors does not provide in our preliminary investigations any evidence of differences between the pathogenic and non-pathogenic strains. Evidently more studies on this subject are needed to solve the problem of a branched electron transport system in these amoebae.

Since the aerobic or anaerobic fermentations were found to be predominant energy-yielding pathways of all endoparasites (Gutteridge and Coombs 1977), even when oxygen concentrations are not a limiting factor (e.g., trypanosomes) the determination of some major enzymes of a modified glycolytic pathway (LDH, CO_2-fixing enzymes and some Krebs cycle enzymes) in amphizoic amoeba was of particular interest.

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Only the malate dehydrogenase activity was in the range of activity of this enzyme in mammalian tissues, being equal in pathogenic and non-pathogenic amoeba strains. Very low activity of the other Krebs cycle enzyme: SDH (17 times lower than in rat heart — Boczoń, unpublished results) may speak, however, for a relative unimportance of some Krebs cycle reactions as an energy source pathway. Fumarate reductase activity, too, was unexpectedly low, but it is worth mentioning that fumarate reductase activity was measured aerobically and this, together with the well known instability of this enzyme in crude homogenates, may not have given a true indication of the ability of the enzyme to produce succinate *in vivo*.

The activity of all the tested enzymes was higher in pathogenic *Acanthamoeba* strain (309) than in non-pathogenic strain (Neff), parallel the higher bioenergetic activity in this strain observed in our bioenergetic studies described above. It does not seem to be true in *Naegleria* strains, where the opposite situation occurs. Some conformation of the difference between the strains of these two genera come from Hadaś's results (1982). In his work the trypsin activity was about 30 times higher in the pathogenic *Acanthamoeba* strain (309) than in non-pathogenic (Neff), while this activity was just slightly higher in the pathogenic *Naegleria* strain (Vítek) than in non-pathogenic (5D). This difference was even more pronounced in trypsin-like activity of the virulent *Acanthamoeba* strains (50% higher activity) obtained from cysts in comparison to the attenuated strains (Kasprzak et al. in press). It is hard to explain the difference between the two genera at the moment, except that the strain 5D, which was considered as our model non-pathogenic *Naegleria* strain, in biochemical (Hadaś and Kasprzak in press) studies proved to be enzymatically atypical (viz: the alpha-glicosidase, beta-galactosidase and pepsin activity in 5D were similar to other *Naegleria* non-pathogenic strains, but the trypsin activity was similar, for a change, to the pathogenic strains and the isozymic esterase and phosphatase pattern was completely different from those of typical pathogenic and non-pathogenic strains).

The results of the investigations on the lytic enzymes and some key enzymes of the bioenergetic metabolism presented in this paper suggest a higher general metabolic activity in all pathogenic *Acanthamoeba* strains. The enzymatic differences within *Naegleria* strains are much less evident and relate to the cytochrome oxidase activity and substrate saturation curves of the decarboxylating malate dehydrogenase only. It is known, however, that the virulent properties of some environmental *Naegleria* strains as measured by the number of dead mice may be variable and of unstable character.

A relatively low activity of PEPCK, one of the CO_2-fixing enzymes and a very low LDH activity (about 40 times lower than in rat liver) may suggest the importance of the other CO_2-fixing enzyme: decarboxylating malate dehydrogenase, which in most organisms studied is thought to decarboxylate malate and seems to be the one subject to a great variety of regulatory properties. Decarboxylating malate dehydrogenase, much more active than PEPCK in all amoeba strains investigated by us, seems to be a better candidate for CO_2-fixing reaction and be-

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cause of its potential regulatory properties it may affect the aerobic fermentation in amoeba, as it was presented in *Critidia fasciculata* (Marr 1973, Orellano and Cazullo 1981) or *Trypanosoma cruzi* (Cannata et al. 1979). The decarboxylating malate dehydrogenase partially purified by Cazullo et al. (1977) in *T. cruzi* showed, in contrast to our results on amoeba, NADP-specificity and activation by Mn^{++} rather and very little by Mg^{++}, and was, like in our experiments, inhibited by oxaloacetate.

The decarboxylating malate dehydrogenase in all amoeba strains investigated by us was like the same enzyme in *Ascaris* of higher NAD than NADP specificity (Langsperger and Harris 1976).

Interestingly, the substrate saturation curves of NAD-specific decarboxylating malate dehydrogenase showed the so called "double sigmoidicity" in pathogenic strains only. This shape might result, as suggested by Kurganov (1975), from a combination of the hyperbolic shape of substrate saturation curve, characteristic of the monomeric enzyme, and the sigmoidal shape, characteristic of enzymes with "frozen" subunits in which the dissociation is very slow and hindered.

The stimulation by fumarate — a positive modulator — particularly evident in decarboxylating malate dehydrogenase from the pathogenic *Naegleria* strain Vítek, supports to some extent the suggestion about the regulatory properties of these enzymes in pathogenic strains only. It is important to stress, however, that these observations, obtained using crude amoeba homogenates of all strains, may be indicative of the potential regulatory properties of the enzyme, but for further confirmation a continuation of those investigations on preparations purified to electrophoretic homogeneity is still necessary.

In conclusion it could be stated that the generally higher metabolic activity in pathogenic amoeba strains than in non-pathogenic ones, suggested by Lastovica on the basis of his ultrastructural observations, was proved in our biochemical investigations on *Acanthamoeba* strains. The regulatory properties of decarboxylating malate dehydrogenase in *Acanthamoeba* and *Naegleria* pathogenic strains only, may suggest also a greater adaptive capability of the pathogenic ones. For those amoeba primarily free-living in the environment the adaptation for survival in a new biotop, i.e. host tissues, is necessary, but those tissues (as lung or brain) are well oxygenated. Therefore the adaptation may be limited to certain enzymes of the bioenergetic metabolis, but the presence of some enzymes with potential regulatory properties (decarboxylating malate dehydrogenase) in pathogenic strains only, may be indicative of their better metabolic adaptation to the new biotops.

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I express my thanks to Prof. Dr W. Kasprzak and Dr T. Mazur for a generous supply of amoeba strains and to Mrs. Krystyna Szeląg, Mrs. Maria Mikołajewska and Mrs. Alicja Matias-Guzek for the supply of amoeba cultures. I also wish to thank Prof. Dr W. Kasprzak for his interest in this study and his helpful advice.

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Contribution à la connaissance des Thécamoebiens des dépôts lacustres

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Synopsis. Les dépôts vaseux lacustres, semblent être le biotope préférentiel des Thecamoebiens de grande taille. L'étude de nombreux prélèvements de pièces d'eau de surface importante, nous permet de décrire trois nouvelles espèces caractéristiques de ce biotope particulier.

D'une façon générale, le benthos des grandes pièces d'eau est un biotope très particulier, constitué de sédiments légers formant ce qui peut être considéré comme des vases sapropéliques au sein desquelles, les matières organiques sont progressivement dégradées par les Bactéries.

Ces dépôts lacustres d'épaisseur variable sont dans leur ensemble constitués des plus fines particules minérales, les micromorpholites, de particules organiques et de squelettes de Diatomées en plus ou moins grand nombre.

Beaucoup de grandes espèces de Thécamoebiens, semblent sinon inféodées, au moins préférer ce type de biotope.

L'ensemble des études fauniques publiées, permet de reconnaître plusieurs faunes typiques: faune des Sphaignes, faune des Mousses, faune des grands fonds, faunes sapropéliques spéciales aux mares et fossés, auxquelles nous pouvons ajouter la faune des vases sapropéliques des grands lacs et étangs peu profonds.

L'étude de lacs et grands étangs de Belgique (van Oye 1953, Chardez 1980, 1985, Chardez et Gaspar 1976, 1982, 1984), nous a révélé outre la présence de populations caractéristiques de ce milieu, la présence de trois espèces nouvelles objet de cette note.

Protocucurbitella mitrata sp. nov. (Pl. I 1, 2)

Diagnose: Thèque large, de section circulaire, à flancs arrondis s'accuminant vers le fond en une pointe plus ou moins prononcée.

Le pseudostome circulaire est bordé d'éléments pierreux formant une denticulation irrégulière, sous laquelle en vue apicale on apperçoit une ébauche de diaphragme laissant une ouverture d'un très grand diamètre.

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Le revêtement est formé de plaquettes minérales polymorphes peu débordantes.

Dimensions: Hauteur 166—190 μm, diamètre 127—130 μm, diamètre du pseudostome 75—84 μm.

Locus typicus: Sapropèle vaseux, fond des grandes pièces d'eau (Virelles, Hainaut, Belgique).

Holotype: n° V7.

Difflugia longum sp. nov. (Pl. II 7, 8)

Diagnose: Thèque très allongée, de section circulaire, terminée en pointe aiguë quelquefois légèrement incurvée sur le côté, la partie de la thèque la plus renflée se situe toujours aux environs du tiers antérieur.

Le pseudostome est circulaire, il est précédé d'une ébauche de col très court, plus ou moins prononcé, mais toujours présent.

Le revêtement est constitué de plaquettes minérales polymorphes plates, plus petites vers le fond de la thèque.

Les pseudopodes de type lobés sont larges et peu timides.

Le cytoplasme est claire, Noyau unique et sphérique d'un diamètre de 28 à 30 μm.

Dimensions: Hauteur 256—280 μm, diamètre 75—86 μm, diamètre du pseudostome 44—47 μm, hauteur du col 3—7 μm.

Locus typicus: Sapropèle vaseux du fond des lacs et étangs (Virelles, Hainaut, Belgique)

Holotype: n° V9.

Difflugia foissneri sp. nov. (Pl. I 3, II 5—7)

Diagnose: Thèque trapue, section transversale ovale, fortement comprimée vers le fond de la thèque, les flancs convergent vers un pseudostome plus ou moins circulaire, le fond de la thèque est muni de trois cornes triangulaires régulières et régulièrement espacées a bout fermé. Ces cornes sont toujours placées dans un même plan. Certains individus ne possèdent que deux cornes parfaitement formées entourant l'ébauche de la corne centrale.

L'ensemble du revêtement est formé de plaquettes minérales transparentes peu débordantes.

Le pseudostome est entouré des mêmes plaquettes formant un bord déchiqueté sans col.

Cytoplasme claire, finement ponctué, pseudopodes de type lobé, souvent unique. Noyau unique sphérique de 30 μm de diamètre.

Dimensions: Hauteur 210—230 μm, largeur 170—180 μm, épaisseur 90—100 μm, diamètre du pseudostome 86—90 μm, longueur des cornes 19—21 μm.

Locus typicus: Sapropèle vaseux du fond des grandes pièces d'eau (Virelles, Hainaut, Epioux, Ardenne, Belgique).

Holotype: n° V8.

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Le nom de cette espèce, est un hommage au Dr. W. Foissner pour sa contribution à l'étude des Protozoaires.

Si nous relevons les espèces habituellement compagnes de ces nouveaux taxons, dont la taille moyenne se situe autour de 200 μm, nous trouvons: *Difflugia urceolata* Carter, *D. corona* Wallich, *D. labiosa* Wailes, *D. maxilabiosa* Chardez, *D. smilion* Thomas, *D. beyensi* Chardez, *D. pyriformis* Perty, *D. gigantea* Chardez, *D. acuminata* Ehrenberg, *D. acuminata magna* Deflandre, *D. giganteacuminata* Chardez, *D. distenda* Ogden, *D. viscidula* Penard, *D. finstertaliensis* Laminger, *D. acutissima* Deflandre, *D. amphoralis* (Leidy) Hopkinson, *D. curvicaulis* Penard, *D. nodosa* (Leidy) Chardez, *Zivkovicia compressa* Ogden, *Z. sarrazinensis* Chardez, *Z. flexa* (Cash et Hopkinson) Ogden.

Nous constatons que toutes ces espèces de grande taille, se rencontrent souvent en populations dans le sédiment des masses aquifères importantes.

SUMMARY

The muddy lacustrian settlings seem to be the preferential biotop of very tall Testate Amoebae. The study of many samplings from large surfaced sheets of water allows us to describe three new characteristic kinds of this particular biotop.

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EXPLICATION DES PLANCHES I–II

1 et 2: *Protocucurbitella mitrata* sp. nov. (×400)
3: *Difflugia foissneri* sp. nov. (×400)
4: *Difflugia longum* sp. nov. (×400)
5: *Difflugia foissneri* sp. nov.
6: *Difflugia foissneri* (vue de profil)
7: *Difflugia foissneri* (variante)
8: *Difflugia longum* (deux variantes)
9A: *Protocucurbitella mitrata* (vue apicale)
9B: *Protocucurbitella mitrata* (détail du pseudostome, montrant l'ébauche du diaphragme)

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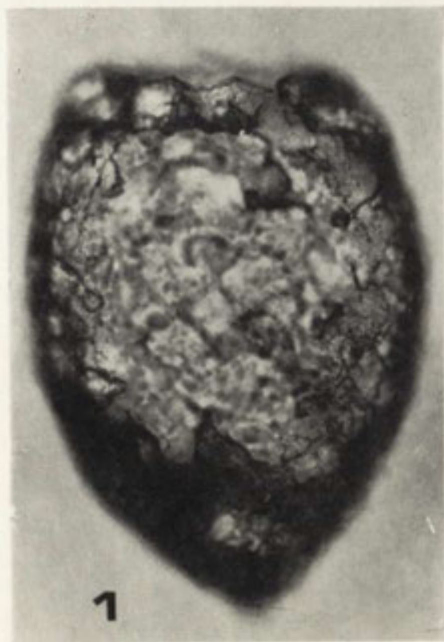

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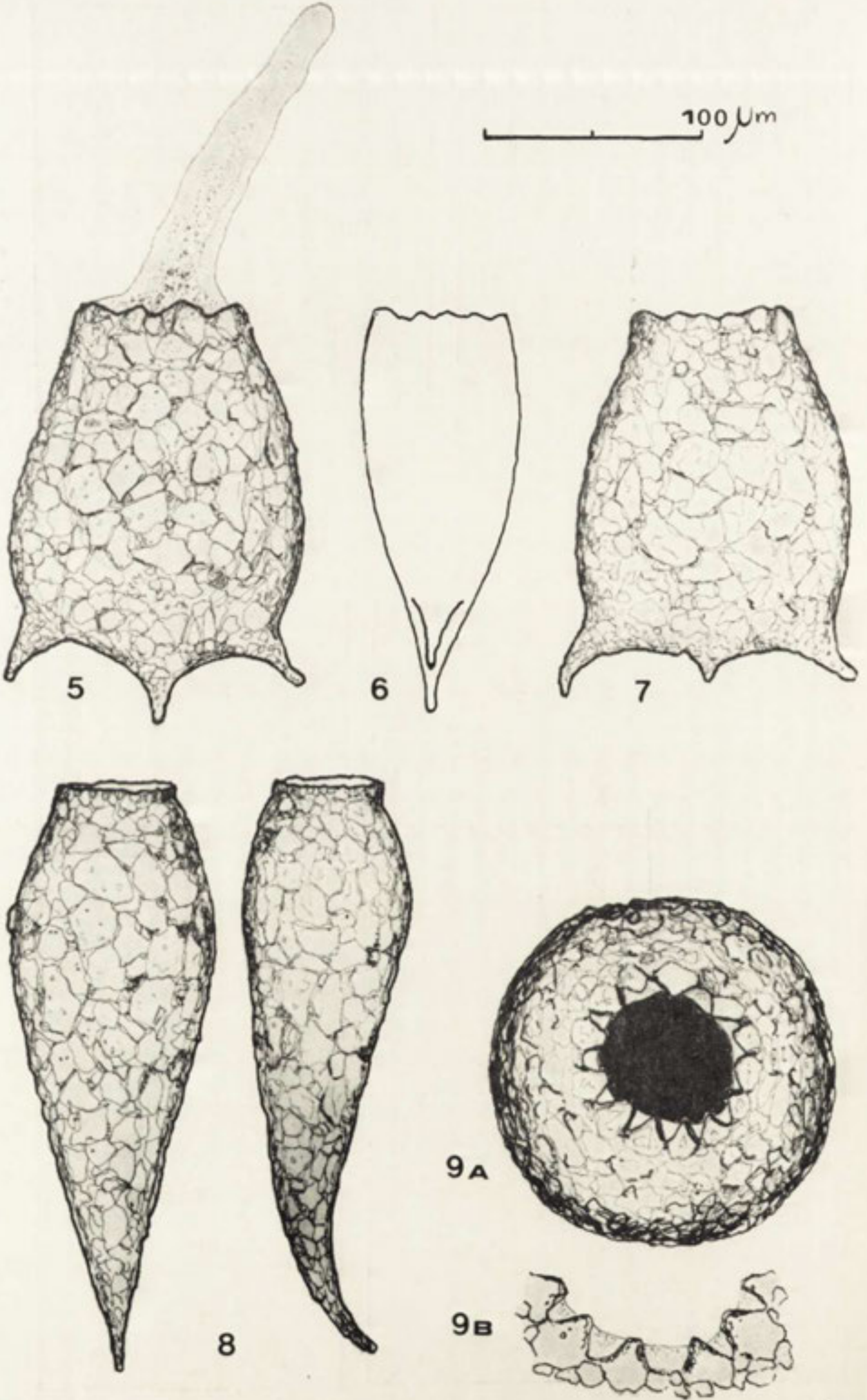

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Trypanosoma balithaensis sp. n. from a Pond Water Turtle, *Lissemys punctata punctata* (Bonnaterre) and Its Development in the Leech Vector *Helobdella nociva* Harding

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Synopsis. This paper represents the description of a new monomorphic trypanosome, *Trypanosoma balithaensis* sp. n. (*Trypanosomatidae*) from a common pond water turtle, *Lissemys punctata punctata* (Bonnaterre) collected from Bankura district, West Bengal, India. The morphology of the trypanosome has been described and compared with other known chelonian haemoflagellates. The life-cycle of this trypanosome in a leech vector *Helobdella nociva* Harding has also been studied in detail.

The paper deals with the description of a new haemoflagellate from a common pond water turtle, *Lissemys punctata punctata* (Bonnaterre) along with its development in a Rhyncobdellid leech vector, *Helobdella nociva* Harding collected from Bankura district, West Bengal, India. Walliker (1965) listed 12 species of chelonian trypanosomes from the world. Subsequently, Sinha (1978) added another new trypanosome, *Trypanosoma gangetica* from *Trionyx gangeticus* Cuvier for the first time from India. This is the second report of its kind from a new host from India along with its life-cycle in a leech vector.

Material and Methods

The turtles were collected during fishing in a pond in the village Balitha of district Bankura, West Bengal, India. Thin and thick blood films were prepared from the blood obtained by nicking the head and feet of the turtles with a scalpel.

The blood films were then air-dried, fixed in absolute methanol and stained with Romanowsky's stains. Organ imprints were also made from the liver, heart, kidney, lung, bone-marrow etc. and fixed and stained in the same manner as the blood films.

Rhyncobdellid leeches, *Helobdella nociva* Harding were found congregate in a considerable

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number on the neck region, round the anal aperture and on the feet of the turtles. They were removed from the turtles and maintained in aquaria or glass vials for several weeks. Ten of them (youngs) were allowed to refeed. Some of the engorged leeches were sacrificed and smears of the contents of the gastric and intestinal caeca were prepared. Citrated saline solution was also used for dilution of the crop or caecal content (3:1) to study the live specimens. Air dried smears were fixed and stained with Romanowsky type of stains. The morphometric parameters were recorded after Hoare (1972).

The type slides will be deposited in the National Collection of the Zoological Survey of India, Calcutta.

Observations

Trypanosoma balithaensis sp. n.
(Figs 1 *1*, *2* and 2 *1–12*)

Type-host: *Lissemys punctata punctata* (Bonnaterre)
Type-locality: Balitha, Bankura, West Bengal, India
Site of infection: Blood
Prevalence: Of 5 turtles examined, 2 were found to be infected
Vector: *Helobdella nociva* Harding

Description

The trypanosomes were monomorphic, elongated with drawn out anterior and posterior end (Fig. 1). They measure 30–35 μm (mean 32.5 μm) in length (PA) excluding free flagellum and 1.5–2.5 μm (mean 2.0 μm) in body width (BW).

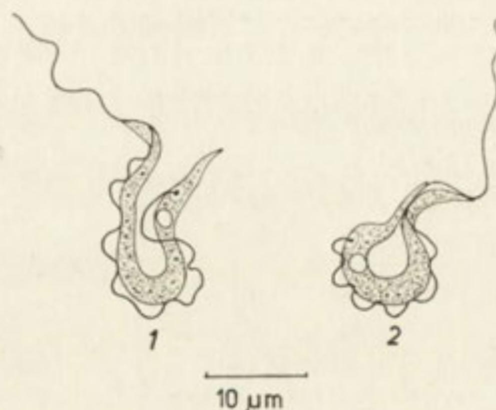

Fig. 1. Camera lucida drawings of *Trypanosoma balithaensis* sp. n.

The free flagellum (FF) ranged from 17.5–20.0 μm with a mean of 18.5 μm. The nucleus was almost round (2.0×2.0 μm) and always situated very close to the kinetoplast (KN = 2.8–3.0 μm) near the posterior end. It stained purple with Leishman's stain. The dot-like kinetoplast was situated a little away from the posterior

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end (PK = 9.2 μm) and always surrounded by a halo. Cytoplasm was homogeneous, granular and without any striations and vacuoles. It stained blue with Leishman. Flagellum was found to emerge from the kinetoplast and had 5 to 6 attachments with the undulating membrane proper and left as a distinct free flagellum.

No divisional stages in the vertebrate hosts were observed.

Development in the Leech Vector (Fig. 2 *1–12*)

A number of leeches which inhabited the body of those turtles were also found naturally infected with this trypanosome.

The crop smears and caecal contents of the leeches *Helobdella nociva* revealed a large number of developmental stages. They were studied and categorized as amastigote, spheromastigote, epimastigote and metacyclic trypanosomes. Besides these, some transitional forms were also encountered. The noted morphological changes were involved during the transition of the vertebrate forms to invertebrate forms in the leech vector.

After two days of infection in the crop of the leech the large vertebrate form became retracted in various ways to form amastigote stage (Fig. 2). They are either round or spherical bodies having prominent dot-like kinetoplast and a nucleus situated either centrally or peripherally. They measure 7.5×7.5 μm. The amasti-

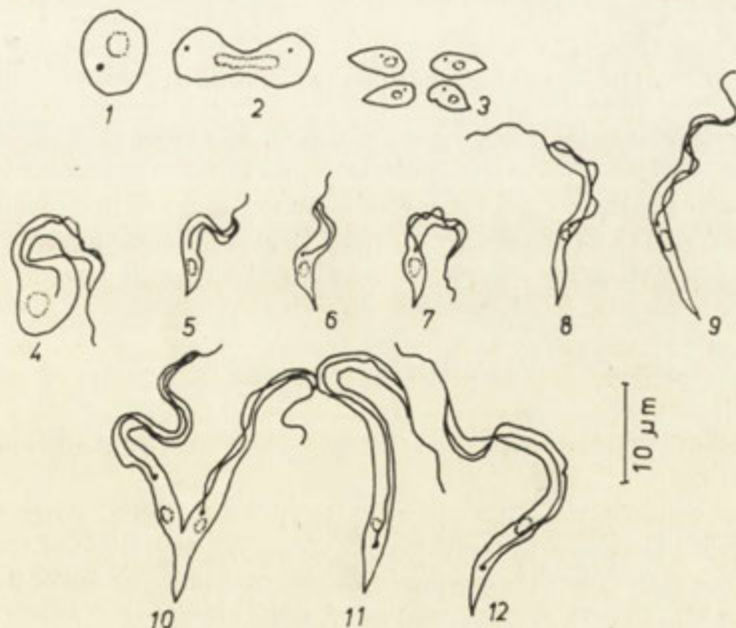

Fig. 2. *1–12* – Developmental stages of *T. balithaensis* sp. n. in the Leech Vector *Helobdella nociva*, *1* – amastigote stage, *2* – binary fission of amastigote stage, 3 – four pyriform amastigote stages produced by binary fission, *4* – spheromastigote stage, *5–7* – short-slender epimastigote forms, *8*, *9* – long-slender epimastigote forms, *10* – longitudinal binary fission of epimastigote stage, *11* – transitional form, *12* – metacyclic trypanosome

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gote form, by means of two successive divisions, produces four small pyriform bodies (Fig. 2 *3*). Some of them developed a short prolongation of the body cytoplasm bordering by flagellum. These are spheromastigote forms (Fig. 2 *4*). They measure 30 µm in total length and 8.5 µm in width with a free flagellum of 4.0 µm. The nucleus is round and very close to the dot-like kinetoplast.

After four days some of the pyriform amastigote stages transformed into epimastigote forms. These are of two types — long slender epimastigote (Fig. 2 *8*, *9*) and short-stumpy epimastigote (Fig. 2 *5—7*). The long slender epimastigotes measure 25.5 µm in length and 2.0 µm in width with a free flagellum of 6.2 µm. The short stumpy forms measure 15.5 µm in length and 3.5 µm in width with free flagellum of 3.5 µm. Some divisional forms of this epimastigote stages have also been observed (Fig. 2 *10*).

After six days the long slender forms became more attenuated with the posterior end pointed (Fig. 2 *11*). The kinetoplast shifted at the posterior end of the body though not at its extremity (Fig. 2 *11*, *12*). Thus they assume the metacyclic trypanoform stages, whose measurements are as follows: length 30 µm; width 2.5 µm; free flagellum 10.5 µm. By the 9th and 10th days the crop and the intestinal caecum of the leech become thickly populated with large number of long, slender motile epimastigote and metacyclic trypanosomes.

When the leeches feed on a new host the metacyclic trypanosomes are inoculated. Thus they develop into typical trypanosome forms only in the blood of the turtles.

Diagnosis of *Trypanosoma balithaensis* sp. n.

This trypanosome is monomorphic, elongated measuring 32.5 µm in length and 2.0 µm in width. The nucleus is almost round and always situated very close to the kinetoplast. The dot-like kinetoplast is situated a little away from the posterior end and always surrounded by a halo. Cytoplasm is homogeneous, granular and without any striations and vacuoles. The free flagellum is 15.5 µm in length.

Discussion

A review of the literature revealed that there are 13 species of chelonian trypanosomes in the world.

Trypanosoma balithaensis has got some resemblances with *T. gangetica* Sinha, 1978 by being monomorphic, in the body configuration and length of the free flagellum. But it differs from *T. gangetica* by the structure and position of the nucleus and kinetoplast. The cytoplasm of the present species is non-vacuolated (7—10 vacuoles in *T. gangetica*).

The present species differs from *T. vittatae* Robertson, 1908 by being monomorphic. *T. vittatae* is pleomorphic having large, intermediate and short forms. *Trypanosoma balithaensis* has also got some close affinity with *T. chrysemydis* Rou-

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dabush and Coatney, 1937 in the structure, but it differs markedly in the length of the flagellum and structure and position of the nucleus.

The haemoflagellate under report differs from all the previous trypanosomes so far described from chelonians in morphometric measurements and other characteristics in details.

Therefore, a new name *Trypanosoma balithaensis* sp. n. has been proposed after its type-locality, to mark its specific identity.

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Chilogregarina bhatiae sp. n. (*Apicomplexa*:*Eugregarinida*) a New Cephaline Gregarine from a Chilopod *Geophilus* sp.

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Received on 27 May 1986

Synopsis. A new cephaline gregarine *Chilogregarina bhatiae* sp. n. (*Apicomplexa*: *Eugregarinida*) has been described from the intestine of a chilopod *Geophilus* sp. from West Bengal, India. Its epimerite is a non-persistant disc with eight truncated digitiform processes — set upon a short, flat neck; trophozoite and sporadin are elongated and leafy bodies with mean dimension of 213.7 μm × 27.8 μm and 114.4 μm × 18.2 μm respectively.

Among Indian arthropods, insects have been extensively investigated for their gregarine parasites. However, only three species of cephaline gregarines have been reported from Indian chilopods (Mitra and Chakravarty 1937, Chakravarty 1939 and Ganapati and Narasimhamurti 1960). This communication describes a new species of cephaline gregarine from the intestine of a chilopod *Geophilus* sp. from West Bengal, India.

Material and Methods

The chilopods were collected from their normal habitats and were brought back alive to the laboratory where their alimentary canals were dissected out with a drop of 0.5% saline water. The contents of the midgut were carefully examined under the microscope for the parasite. The smears of the infected midgut contents were fixed in Schaudinn's fixative and subsequently were stained with iron alum haematoxylin method. The figures were drawn with the aid of a camera lucida and the measurements were taken in micrometer (μm).

Observations

The very scanty infection of cephaline gregarine was noticed in the intestine of two of the six chilopods that were examined during the investigation.

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Trophozoite

It was flat, leafy body consisting of a terminal non-persistent, discoid, digitiform epimerite; a moderate neck; an ellipsoidal protomerite and an elongated deutomerite which gradually tapered posteriorly to a bluntly pointed end (Fig. 1 *1*).

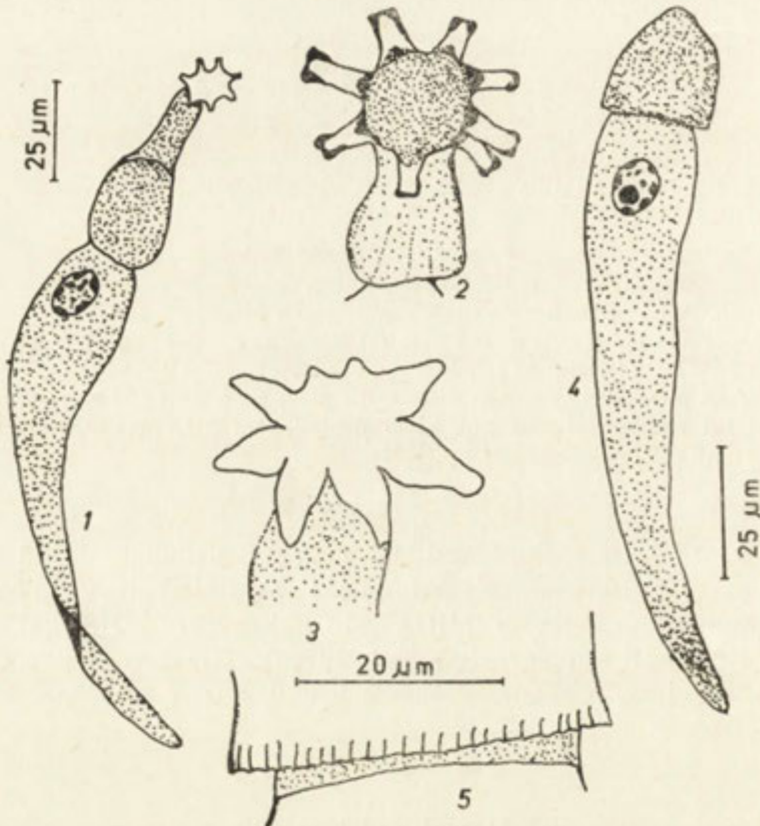

Fig. 1 *1*–*5*. Structures of *Chilogregarina bhatiae* sp. n., *1* — a fully grown trophozoite, *2*, *3* — epimerite with neck enlarged, *4* — a fully grown sporadin, *5* — posterior edge of the protomerite of sporadin showing serrated nature

It had a spherical to ellipsoidal nucleus anteriorly. The epimerite was a complex disc with eight short digitiform processes which were truncated terminally and the terminal edges of each truncated process were thickened (Figs. 1 *2* and *3*). The dimension of the largest trophozoite was 226.0 µm × 28.0 µm.

Sporadin

The sporadins were small, solitary, leafy, elongated bodies consisting of anterior invertedly cup-like protomerite and elongated deutomerite tapering posteriorly to a bluntly pointed end (Fig. 1 *4*). The posterior edge of the protomerite facing the protomerite-deutomerite septum was highly serrated (Fig. 1 *5*). The ellipsoidal to spherical nucleus with a small, round karyosome was situated at the anterior

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end of the deutomerite. The dimension of the largest sporadin was 198.0 μm × 25.0 μm.

Gametocyst and sporocyst were unkown.

Measurements

Table 1

The detail measurements (μm) of the different parts of the trophozoite and sporadin of *Chelogregarina bhatiae* sp. n. are given below:

Sl. No.	TL	DE	LNc	LP	WP	LD	WD	LN	WN	WP:WD	LP:TL
(1)	192.0	15.0	16.0	28.0	20.0	140.0	28.0	12.0	8.0		
(2)	223.0	15.0	10.0	32.5	25.0	175.0	27.5	16.0	14.0		
(3)	226.0	12.0	12.0	28.0	26.0	180.0	28.0	15.0	11.0		
(4)	74.0			14.0	12.0	60.0	16.0	8.0	8.0	1:1.33	1:5.3
(5)	82.0			16.0	12.0	66.0	16.0	10.0	10.0	1:1.33	1:5.1
(6)	98.0			18.0	13.0	80.0	16.0	12.0	10.0	1:1.23	1:5.4
(7)	120.0			20.0	15.0	100.0	18.0	12.0	10.0	1:1.20	1:6.0
(8)	198.0			30.0	21.0	168.0	26.0	15.0	10.0	1:1.19	1:6.6

WP: WD = 1:1.19–1.33 (1.26), LP:TL = 1:5.1–6.6 (5.7)

Abbreviations: TL – Total Length, DE – Diameter of Epimerite, LNc – Length of Neck, LP – Length of Protomerite, WP – Width of Protomerite, LD – Length of Deutomerite, WD – Width of Deutomerite, LN – Length of Nucleus, WN – Width of Nucleus, WP:WD – Ratio of Width of Protomerite to Width of Deutomerite and LP:TL – Ratio of Length of Protomerite to Total Length.

Discussion

The cephaline gregarine is assigned to the genus *Chilogregarina* Levine, 1979 since it is reported from a chilopod host and it possesses a non-persistent discoid digitiform epimerite at the apex. While proposing the genus *Chilogregarina*, Levine (1979) included four species of cephaline gregarines viz., *Chilogregarina striata* (Léger et Duboscq, 1903) Levine, 1979. Type species reported from the intestine of *Scolopendra cingulata*; *C. brasiliensis* (Pinto, 1918) Levine, 1979 reported from the intestine of *Scolopendra* sp.; *C. stella* (Léger, 1894) Levine, 1979 reported from the intestine of *Geophilus grabielis* and *C. dujardini* (Schneider, 1875) Levine, 1979 reported from the intestine of *Lithobius forficatus*. The present gregarine agrees closely with *Chilogregarina stella* (Léger, 1894) Levine, 1979 in having eight processes in the epimerite and closely related length of the trophozoite and sporadin. However, the parasite in study differs from the latter by short and truncated digitiform processes of the epimerite and serrated posterior edge of the protomerite of sporadin and finally the flat, leafy body of the trophozoite and sporadin. Considering the above distinct features, I believe it is a new species for which the name *Chilogregarina bhatiae* sp. n. is proposed after Prof. B. L. Bhatia who pioneered the research on gregarines in India.

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Material

Holotype and paratypes on slide No. CHIC-3, prepared from the midgut content of *Geophilus* sp. collected from Chinsurah, West Bengal, India are deposited in the Department of Zoology, R. B. C. College, Naihati 743165.

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Hepatozoon darjeelingensis sp. n. from the Himalayan Flying Squirrels

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Received on 14 February 1986

Synopsis. Hepatozoon darjeelingensis sp. n. (*Adeleina*:*Hepatozoidae*) a non-pigmented haematozoa is described from the blood and organ of the Himalayan flying squirrels, *Petaurista magnificus* (Hodgson) and *Petaurista nobilis nobilis* (Gray) (*Rodentia*:*Sciuridae*) collected from Darjeeling (altitude 2000–2010 m MSL), West Bengal, India. In the peripheral blood, gametocytes are exclusively intra-leucocytes. Trophozoites and early schizonts are found in the spleen sections. The present species is compared with the allied species and is considered to be a new species which is reported for the first time from the Indian sub-continent.

Hepatozoa are recorded from the peripheral blood of a number of mammals, including the ground squirrels (Wenyon 1926, Clark 1958), but there is scanty report of their occurrence from the Himalayan flying squirrels. In India, an undescribed *Hepatozoon* from the flying squirrel *Petaurista petaurista* (Pallas) was first recorded by Donovan (cited by Wenyon 1926). After a lapse of many years, Dasgupta (1965) observed a *Hepatozoon* in the leucocytes of Hodgson's flying squirrel, *Petaurista magnificus* (Hodgson) collected in Darjeeling (altitude 2000 m MSL), West Bengal. Sinha (1984) also studied the same parasite in *Petaurista nobilis nobilis* (Gray) obtained from the same locality.

This communication deals with the description of a new species of *Hepatozoon* of two North eastern Himalayan flying squirrels, *Petaurista magnificus* (Hodgson) and *Petaurista nobilis nobilis* (Gray) collected from Darjeeling (altitude 2000–2010 m MSL), West Bengal, India.

Material and Methods

Four specimens (*Petaurista nobilis nobilis* Gray) were examined in the laboratory. Two of these were found to harbour *Hepatozoon* in their peripheral blood. Blood was obtained by puncturing the brachial vein of the fore-arm and stained with Leishman's and Giemsa's stains. Small

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pieces of tissue from liver, lung and spleen were fixed in Bouin's and Carnoy's fixatives and sectioned. These were stained in haematoxylin-eosin and Heidenhains iron-haematoxylin stains. Measurements of parasites were made from fixed and stained materials and camera lucida drawings were made.

Results

Hepatozoon darjeelingensis sp. n.

Type host: *Petaurista magnificus* (Hodgson) and *Petaurista nobilis nobilis* (Gray)
Type locality: Darjeeling (altitude 2000–2010 m MSL), West Bengal, India
Site of infection: Peripheral blood and spleen

Morphology

In the circulating blood, parasites occurred in the form of intra-leucocytic gametocytes. No extra-corpuscular form was seen.

Gametocytes (Fig. 1 *1–6*)

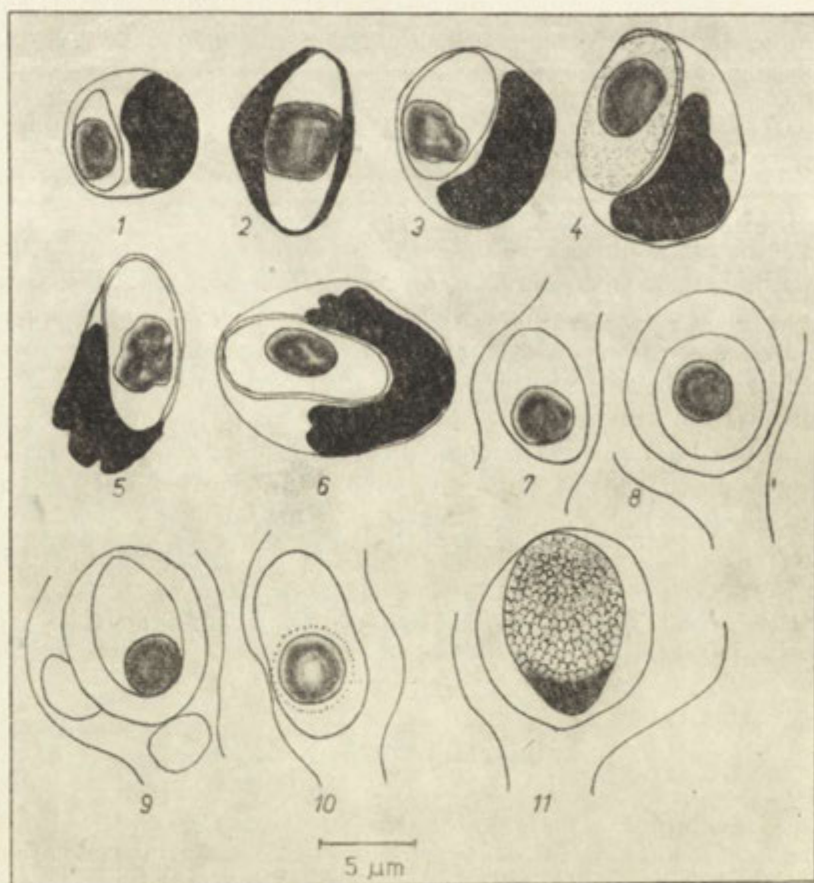

Fig. 1. *1–11*. Camera lucida drawings of *Hepatozoon darjeelingensis* sp. n. *1–6* – Gametocytes, *7, 8, 10* – Trophozoites, *9, 11* – Early schizonts, (*7–11* in tissue sections of spleen)

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These were few in blood smears. Depending on their size, shape and staining properties only single morphological type was detected. Young forms (Fig. 1 *1–3*) were either oval or slightly bent at one pole measuring 11–13×4–5.5 μm (average 12×4.2 μm). Cytoplasm was non-granular, sometimes vacuolated or not and stained light pink in colour. Nucleus was central or sub-central, slightly oval and stained deep pink with an average measurement of 6×4 μm. Mature forms (Fig. 1 *4–6*) were capsulated, broad and oval measuring 13.2–14.5×5.8–7.5 μm (average 13.5×6.3 μm). Cytoplasm was homogeneous, granular or non-granular and stained very faintly. Nucleus was elongate or oval with irregular outline and stained deep pink. The average measurement of the nucleus was 7×4 μm.

Tissue phase

No divisional stages were encountered in peripheral blood smears. Parasites showing early development in the spleen were, however, found in the sections of spleen.

Trophozoites (Fig. 1 *7, 8, 10*) were either ovoid or spherical measuring 5–9× ×2.4–7.8 μm (average 7×4.4 μm). Cytoplasm was homogeneous, non-granular, non-vacuolated and took up light eosin stain. Nucleus was round, terminal or sub-terminal, stained deep blue with haematoxylin. Sometimes an unstained area occurred around the nucleus (Fig. 1 *10*). The average measurement of the nucleus was 3 μm in diameter. A vacuole was also present around some trophozoites. Early schizonts (Fig. 1 *9, 11*) were large and ovoid in shape measuring 10–12×5–8 μm (average 11.2×6.5 μm). Cytoplasm was foamy in appearance and stained light pink with eosin. Nucleus was elongated and with dispersed chromatin, indicating the initial stage of division, terminal in position and stained deep blue with haematoxylin (Fig. 1 *11*).

Invasion of this parasite caused hypertrophy of infected host cells. Usually nucleus of leucocyte was markedly displaced to one side. Shape of hypertrophied infected cells depended on the position of parasite and on the displaced host cell nucleus. In some, host cell nucleus was seen in fragmented condition.

Diagnosis of *Hepatozoon darjeelingensis* sp. n.

Gametocytes are exclusively intraleucocytic and exhibiting single morphological type, measuring 11–14.5×4–7.5 μm. Development is in the spleen of host flying squirrel in the form of trophozoites and early schizonts measuring 5–9× ×2.4–7.8 μm and 10–12×5–8 μm respectively.

Discussion

Wenyon (1926) considered all the haemogregarines of mammals in the genus *Hepatozoon* Miller, 1908. Levine et al. (1980) placed the genus *Hepatozoon* in the family *Hepatozoidae* of the sub-order *Adeleina* under the class *Sporozoa*. Schi-

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Table 1

Comparative study of *Hepatozoon funambuli* (Patton 1906, 1908), *Hepatozoon petauri* (Welsh and Barling 1909, 1910), *Hepatozoon sciuri* (Coles 1914), *Hepatozoon griseisciuri* Clark (1958) and the present parasite *Hepatozoon darjeelingensis* sp. n. (measurements in micrometers, μm)

Specification	*H. funambuli*	*H. petauri*	*H. sciuri*	*H. griseisciuri*	*H. darjeelingensis* sp. n.
Gametocytes (intra-corpuscular form)	One type 10×5	One type 5−8×2.5−4	Two types Oval 10×4.5 Curved 16−18×3	One type 9.9−11.8×3.3−4	One type 11−14.5×4−7.5
Nucleus of gametocytes	—	—	Oval 6−4.5 Curved 8−3	4.3.3×5.2−3.3	5−10.5×3−5
Development in vertebrate host	Unknown	Unknown	In liver and lung tissues, on the heart blood	In liver and spleen tisues, bone-marrow	In spleen tissues
Types of tissue schizonts	Unknown	Unknown	One type 25×18.7	Two types 16.5−23.1×13.2−18.4 15×8	One type 10−12×5−8
Vector*	Unknown	Unknown	*Orchopeas wickhami*	*Euhaemogamasus ambulans* *Echinolaelaps echidninus*	Unknown
Site of infection	In leucocytes of peripheral blood	In erythrocytes of peripheral blood	In leucocytes of peripheral blood	In leucocytes of peripheral blood	In leucocytes of peripheral blood
Vertebrate host*	Palm squirrel, *Funambulus pennantii* Wroughton	Marsupial flying squirrel, *Petaurus norfolcensis* Kerr *P. breviceps* Waterhouse	ground squirrel, *Sciurus vulgaris* Linnaeus	grey squirrel *Sciurus carolinensis* Gmelin	Himalayan flying squirrel *Petaurista magnificus* (Hodgson) *P. nobilis nobilis* (Gray)
Locality	Bombay, Kathiawar, India	Sydney, Brisbane, Australia	England	Eastern Maryland, United States	Darjeeling, West Bengal, India

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zogony in the spleen of flying squirrel has placed the present parasite under the genus *Hepatozoon*.

Parasites from blood smears of *P. magnificus* previously prepared by one of us (Dasgupta 1965) were compared with those found in the blood smears of *P. nobilis nobilis*. All the parasites thus studied were considered identical and identified as *Hepatozoon darjeelingensis* sp. n. on the basis of the above characters.

Clark (1958) listed six named and three unnamed species of *Hepatozoon* in the family *Sciuridae*, of which only one undescribed species of *Hepatozoon* was recorded from flying squirrel (Wenyon 1926) in India. Two more unnamed species of *Hepatozoon* were described by Dasgupta (1965), Sinha (1984) and Sinha and Dasgupta (1986) from Himalayan flying squirrels. Besides these, *Hepatozoon petauri* (Welsh and Barling 1909, 1910) was also described from a marsupial flying squirrel in Australia. The same parasite was also studied by Mackerras (1959) in *Petaurus norfolcensis* Kerr and *P. breviceps* Waterhouse from Brisbane, Australia.

The present parasite resembles to some extent *Hepatozoon funambuli* (Patton 1906, 1908) as redescribed by Bhatia (1938), *H. petauri* (Welsh and Barling 1909, 1910), *H. sciuri* (Coles 1914) and *H. griseisciuri* Clark (1958) in general configuration of gametocyte infecting the leucocytes, except for *H. petauri*, where infection takes place in the erythrocytes, but differs sufficiently in other morphological characters and in host range as shown in Table 1. It is evident from the comparative study that the present parasite does not completely resemble any of or any other known species of the genus. Moreover, the geographical, hostal and morphometric differences are enough to distinguish the present species from previously named species and it is therefore considered as a new species. The species name of the parasite is given after the name of the type locality. The holotype and paratypes will be deposited in the National Zoological Collection of Zoological Survey of India, Calcutta.

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Zschokkella stettinensis sp. n. (*Myxospora, Bivalvulida*) — a Parasite of Eel, *Anguilla anguilla* (L.)

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Synopsis. New species of a myxosporidian, *Zschokkella stettinensis* sp. n., is described. The parasite was found in the urinary bladder of eel, *Anguilla anguilla* (L.) from Szczecin Bay and Dąbie Lake situated near the estuary of the Odra river.

Protozoans of the genus *Zschokkella* Auerbach, 1910 are parasites of the gall bladder and excretory system of both marine and fresh water fishes. Kudo (1919) gave the descriptions of 4 species: *Z. hildae* Auerbach, 1910, *Z. nova* Klokačewa, 1914, *Z. acheilognathi* Kudo, 1916 and *Z. globulosa* Davis, 1917. Another species, *Sphaeromyxa ovata*, originally described by Dunkerly (1921) from vicinity of Plymouth from marine fishes, has been transferred later by Kudo (1933) to the genus *Zschokkella* under the name *Z. ovata* (Dunkerly) (= *Sphaeromyxa ovata* Dunkerly). Myxosporidians of marine fishes from the vicinity of Plymouth were also studied by Tripathi (1948) who described *Z. russelli* and *Z. sturionis*. Moreover, Chakravarty (1943)[1] found *Z. fossilae* and *Z. ilishae*, and Lalitha Kumari (1969) – *Z. labeonis* and *Z. ophicephali* in fresh water fishes of India. *Z. rovignensis* was found by Nemeczek (1922) in *Scorpaena scrofa* and *S. porcus* from Adriatic Sea (Istria, Italy), while *Z. dogieli* – by Pogorelceva (1964) in mugilid fishes from the Black Sea. *Z. floridanae* was found by Rapacz et al. (1973) in *Floridichthys carpio* from the Biscayne Bay of Florida. In 1976 Moser and Haldorson found *Z. embiotocidis* in marine fishes of the family *Embiotocidae* from the region of California. Then after, Moser and Noble (1977) found 6 species of the genus *Zschokkella* (including 3 new species: *Z. kudoi*, *Z. microcapsula* and *Z. meglitschi*) in macrourid fishes from the Atlantic, Pacific and adjacent seas. Evdokimova (1977) described *Z. flexuosasuturalis* from the urinary bladder of *Paralichthys patagonicus* of the coast of Argentina (south-western Atlantic). In 1966 Šulman gave the descriptions of 8 species of the genus *Zschokkella* from the

[1] Cited after Lalitha Kumari (1969).

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USSR, and in 1984 the same author listed 9 species, 5 of which are not mentioned in the literature mentioned above. These are *Z. orientalis* Konovalov et Schulman, 1966; *Z. parasiluri* Fujita, 1927; *Z. ophiocephali* Chen, 1961; *Z. costata* Kaschkowsky, 1965 and *Z. striata* Schulman, 1962.

The myxosporidians found in *Anguilla anguilla* (L.) differ from all species hitherto described, allocated to genus *Zschokkella*. The observed distinguishing features encouraged the author of this paper to describe these protozoans as a new species, *Zschokkella stettinensis* sp. n.

Material and Methods

The examined eels, *Anguilla anguilla* (L.), were originated from Szczecin Bay (old bed of the Odra river) and Dąbie Lake (joined with the estuary of the Odra river). Out of 37 examined fishes, 24 eels were examined in August 1983 (from Szczecin Bay) and 13 eels — in July and August 1985 (from Dąbie Lake). The total length of examined eels ranged from 39 to 65 cm, their body weight

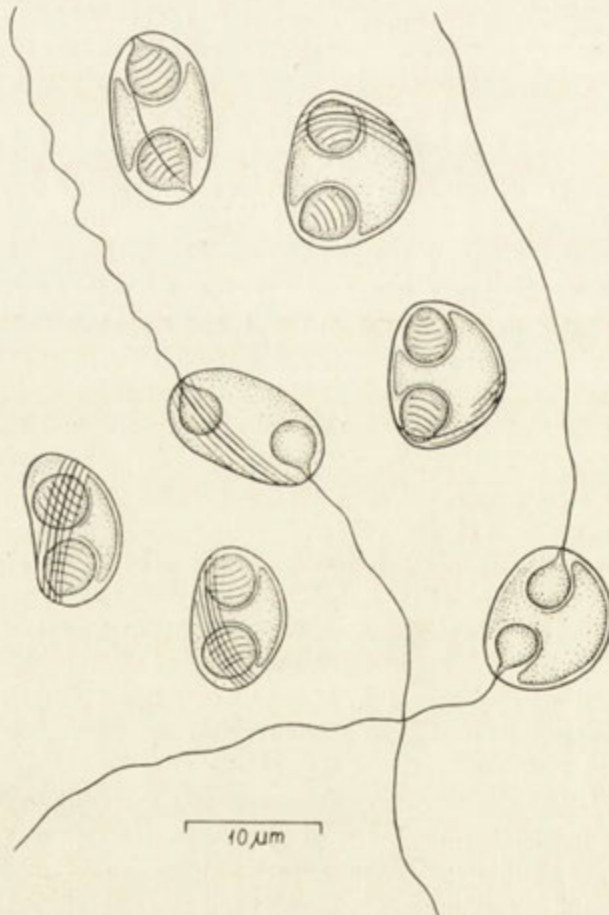

Fig. 1. *Zschokkella stettinensis* sp. n., spores (fresh material)

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was from 100 to 530 g. During dissection, scrapings were taken from the inner surface of the urinary bladder and observed under the microscope. Smears were stained after Pappenheim (May-Grünwald and Giemsa). The drawings of spores were taken from both fresh and stained preparations. Measurements were made on 30 spores from stained preparations.

Description

Zschokkella stettinensis sp. n. (Figs 1, 2)

Host: eel, *Anguilla anguilla* (L.)
Location: urinary bladder
Locality: Szczecin Bay and Dąbie Lake
Infection: spores were found in 18 eels (extensity 48.65%), intensity amounted from single specimens to very numerous spores in a fish

When seen from the suture side the spores are elongated ovals, while in suture plane their shape is more rounded being only slightly greater in length than in width (Fig. 1). One side of the spore, near of which polar capsules are situated, is slightly flattened. The opposite side is rounded. Feebly curved suture line runs slantwise along the spore at a small distance from the poles. At each side of the suture line, parallel to it, two delicate streaks are running along the spore. They are visible only in the fresh material. The polar capsules are almost spherical in shape with short, narrowed ends. Their outlets are situated near the poles, in opposite directions. They contain spirally rolled filament forming usually 5 coils. The sporoplasm occupies mainly the more convex part of the spore; its narrow band penetrates the space between polar capsules. In stained preparations a single large vacuole is visible in the sporoplasm (Fig. 2).

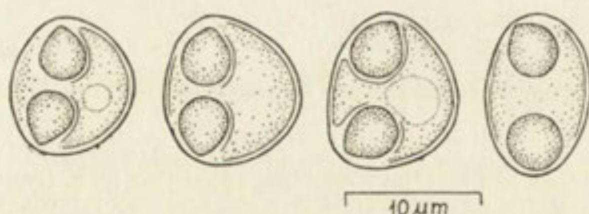

Fig. 2. *Zschokkella stettinensis* sp. n., spores (stained preparation)

Spore dimensions (from stained preparation, in micrometers): length 9.6—11.6 (10.53±0.55)[2], width 7.2—10.0 (8.62±0.53), thickness about 7.6—8.0, width of polar capsules 2.8—4.0 (3.22±0.30), length of polar capsules 3.2—4.4 (3.89±0.33), polar filament length 68—76 (71.67±2.66). After expulsion of polar filaments the polar capsules diminish their size (Fig. 1). Vegetative stages will be described in another paper.

[2] In parantheses: arithmetical mean and standard deviation.

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Discussion

A comparison of morphology of *Z. stettinensis* sp. n. spores found in eel, *A. anguilla*, with descriptions of another species recognized as representatives of the genus *Zschokkella*, has revealed its similarity to *Z. globulosa* Davis, 1917 from the urinary bladder of *Sphaeroides maculatus*. However, this similarity is expressed only in the shape and dimensions of the spores; other characters are distinct. *Z. globulosa* spores have smooth surface in contrast to streaked one in the new species. The suture line in the new species is only feebly twisted and runs at a small distance from the poles, while in *Z. globulosa* the suture line is twisted in relation to the longitudinal axis of the spore (Kudo 1919).

Of the remaining species of the genus *Zschokkella*, two are similar in shape to *Z. stettinensis* sp. n. These are: *Z. microcapsula* Moser et Noble, 1977 from the gall bladder of *Coelorhynchus kishinouyei*, *C. macrolepis* and *C. velifer*, and *Z. meglitschi* Moser et Noble, 1977 found mainly in the urinary bladder, rarely in the kidneys and gall bladder of 11 species of macrourid fishes. However, both these species differ from *Z. stettinensis* sp. n. by lack of streaks and strongly twisted suture line. When the drawings of the species under discussion are compared (cf. Moser and Noble, op. cit.) one can see that the distance between polar capsules in *Z. microcapsula* and *Z. meglitschi* is much larger than in *Z. stettinensis* sp. n. Moreover, the polar capsules of *Z. microcapsula* are about two times smaller than in the new species.

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