PL ISSN 0065-1583

POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA PROTOZOOLOGICA

VOLUME 28

Number 3–4

PAŃSTWOWE WYDAWNICTWO NAUKOWE
WARSZAWA 1989 WROCŁAW

http://rcin.org.pl

POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA PROTOZOOLOGICA

International Journal of Protozoology

Editors

Stanisław DRYL and Stanisław L. KAZUBSKI

Editorial Board

Chairman: Leszek KUŹNICKI
Vice-chairman: Andrzej GRĘBECKI

Members

Magdolna Cs. BERECZKY
Y.-Z. CHEN
Stanisław DRYL
Vassil GOLEMANSKY
Witold KASPRZAK
Stanisław L. KAZUBSKI
Jiři LOM
Dmitrii V. OSSIPOV
Georg Ivanovič POLJANSKY
Igor Borysevič RAIKOV
Ksenia Mironovna SUKHANKOVA

Managing Editor and Editorial Board Secretary

Małgorzata WORONOWICZ

Manuscripts may be submitted to the Editorial Office: Acta Protozoologica, M. Nencki Institute of Experimental Biology, 02-093 Warszawa, 3 Pasteur Street, Poland, or to each member of the Editorial Board.

Subscription orders for all the magazines published in Poland available through the local press distributors or directly through the:

Foreign Trade Enterprise

ARS POLONA

00-069 Warszawa, Krakowskie Przedmieście 7, Poland

Our bankers:
BANK HANDLOWY WARSZAWA S.A.

ACTA PROTOZOOLOGICA appears quarterly. The indexes of the previous volume will appear in No. 1 of the next volume.

Indexed in Current Contents and in Protozoological Abstracts.

http://rcin.org.pl

Jan DEMBOWSKI

Born on 26 December 1889 in St. Petersburg
Died on 22 September 1963 in Warsaw

http://rcin.org.pl

http://rcin.org.pl

Hundred years ago Jan Bohdan Dembowski, one of the Polish leading protozoologists and ethologists was born in St. Petersburg, as a solitary son of Kazimierz Dembowski, an engineer-technologist and Jozefa Dembowska neé Mazurkiewicz. At that time Dembowski's home was the secret center of the Polish Socialist Party in St. Petersburg. Dembowski received M. S. degree from University of Petersburg in 1912, and in the same year became assistant at the Department of Invertebrates Zoology, headed by V. A. Dogel. In 1914 Dembowski continued post graduated studies at the Biologische Versuchsanstalt in Viena. In 1918, Dembowski arrived to Warsaw and moved to the just organized Nencki Institute of Experimental Biology, where he remained until 1934 rising from position of assistant to president. Dembowski married (1918) Wiktoria Stanisława Swinarska (Dembowska), who became his nearest co-worker up to her end (1962). Dembowski was graduated as a Ph.D. at the University of Warsaw (1920) and became a "decent" in zoology at the same University (1922). From 1934 to 1939 he was a professor of Biology of Stefan Batory University in Vilna. Upon the outbreak of World War II all Poles have been removed from the University. From 1939 to 1944 Dembowski survived in Vilna carrying different jobs like a bookssalesman, book-keeper. After Vilna was liberated from Nazi occupation by Red Army and Polish Underground Army (1944), Dembowski went to Moscow, where up to 1947, he was scientific attaché of the Polish Embassy and simultaneously carried out research in the area of protozoology and animal ethology. Upon his return from Soviet Union to Poland Dembowski was in charge of the Nencki Institute, temporarily reestablished in Lodz (Łódź). In addition, he was the professor and head of the Experimental Biology Chair at the University of Lodz (1947–1952). Under Dembowski presidency the construction in Warsaw of new buildings of Nencki Institute was executed (1952–1956) and the Institute became again a main center of the protozoological research in Poland. Pre-war Dembowski's co-workers and followers were either dead or out of Poland. From 1952 to 1963 eleven students of professor Dembowski got the Ph.D. degree. In the period 1948–1957 Dembowski was highly involved in the reorganization process in science and political activity. He prepared and presided over the First Congress of Polish Science (1951). On the basis of the Congress resolution, the Polish Academy of Sciences was founded (1952) and Dembowski was its first President (1952–1957). From 1952 to 1957 Dembowski simultaneously held state offices. He was the President of the Polish Parliament (Sejm) and the Vice-president of the State Council.

Professor Dembowski was a member of international and foreign scientific organizations and societies among them honorary fellow of the Academy of Sciences of the USSR, foreign member of the Hungarian Academy of Sciences and of the National Academy of Science in New York. Dembowski became an emeritus professor in 1961, after his retirement he continued to work regulary at the Nencki Institute until the end.

From 1911 to 1963 he published a great number of scientific papers on subjects that included the eye anatomy of the crab, *Ocypoda ceratophthalama*, the motion mechanism of gregarines, relationships of the coloration of young salamanders with the coloration of the background, behavior of the crabs *Dromia vulgaris*, Uca pugilator and larva of the caddis fly *Molanna angustata*. The most important experimental research concerned exclusively *Paramecium caudatum*. Dembowski at first investigated the phenomenon of a food preference. By the application of an original method of diluting the food suspensions, he showed that *P. caudatum* food intake is two-phasic and that it consists of (1) the automatic and rhythmic

http://rcin.org.pl

actions of swallowing and (2) the ingestion of suspended particles. Furthermore, he found that a considerable concentration of dyes, contained in a fluid, can occur in the course of its intake.

It was also shown by Dembowski that, despite the fact that ciliates have the capability of the food preference, the refusal to ingest some suspensions by *P. caudatum*, even after few days of incubation, cannot be considered a symptom of memory — as believed by Metalnikov — but only the result of a non-specific injury of the ciliate cell body. Likewise, no conditioned response to the boundary of light and shadow could be established in *P. caudatum*. At the same time, on the basis of ingenious experiments, Dembowski succeeded in showing the nature of the error, committed by authors who, in such cases, obtained positive results.

The most important papers concerned the regularities of locomotion and geotaxis in *P. caudatum*. Dembowski, showed in *Paramecium* the fixity of the angle of reflection (about 70°) from a solid obstacle, which allows one to envisage the path of the ciliate as it moves in a vessel of any shape.

On the basis of a series of experiments, Dembowski showed that the center of gravity of *P. caudatum's* body is shifted posteriorly and that the posterior, heavier half of the ciliate's body constitutes a subtle means of its orientation, replacing the sense of equilibrium. Consequently, Dembowski progresively developed a Verworn mechanical hypothesis of geotaxis.

Dembowski did not restrict himself to the experimental research only. He published books and brochures, among others, a classical work in Polish literature of this subject, entitled, "The Natural History of a Protozoan". In general, Jan Dembowski's bibliography consists of over 120 titles, in this number 9 books some of which were published in a few editions and translated into foreign languages. Thus, for instance, "Animal Psychology" appeared in two Polish editions (in 1946 and 1950), one German (in 1955) and one Russian (1959). "Psychology of Apes" was twice issued in Polish (1946, 1951) and once in Italian (1950), German (1956) and Russian (1963).

Despite his honors and recognition, Jan Dembowski was an unassuming, modest man.

Leszek Kuźnicki

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 215-229 (1989)

The Relationship Between Step-up and Step-down Photophobic Responses in *Euglena gracilis*

Leszek KUŹNICKI and Patricia L. WALNE

Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., 02-093 Warszawa, Poland, and Department of Botany and Program in Cellular, Molecular and Developmental Biology, University of Tennessee, Knoxville, Tennessee 37996-1100, U.S.A.

Received on 28th November, 1988

Synopsis. On the basis of studies of step-up and step-down photophobic responses in *Euglena gracilis*, a new hypothesis concerning the mechanisms and molecular processes involved in photoreception and photosensory transduction is presented. It is postulated that the threshold value for the step-up response is determined by: (1) plasmamembrane conduction-resistance properties for phototransduction processes; (2) active chromophore molecules of the a) plasmamembrane photosensitive system and b) of the flagellar swelling; and (3) photoprotective chromophores of the stigma. It is further proposed that in the flagellar swelling flavoproteins exist in two distinct signaling forms: "compact" (folded) and "unfolded". After illumination, a fraction of the flavoproteins, excited by photon absorption, undergoes a conformational change to the compact form. The strength of the step-down response is proportional to the conversion rate from the compact forms to the unfolded forms. The relationship between the step-up photophobic response threshold and the appearance and disappearance of the step-down photophobic reaction reflects the proportion of the two signaling forms of the same chromophore (flavoproteins).

On of the major problems faced in studying light-induced motor reactions of *Euglena gracilis* since 1972 (Diehn 1972) has been the determination of how many photoreceptor chromophores and sensory transduction chains are involved in the photophobic responses (Colombetti et al. 1982; Walne et al. 1984; Colombetti and Petrachi in press). Are there two different photoreceptor systems, in the sense that the step-up and the step-down photophobic responses have different primary chromophores (Diehn 1972; Mikołajczyk and Diehn 1975, 1976, 1978) but a common sensory transduction chain (Mikołajczyk and Diehn 1978; Diehn 1985), or is there only

http://rcin.org.pl

one primary chromophore (i.e., flavins) for both photophobic reactions, each, however, having a separate transduction chain (Checcucci et al. 1976; Diehn 1979; Colombetti et al. 1981, 1982)?

The controversy about photosensory transduction in *E. gracilis* is perhaps more complex than ever, despite a number of experimental studies on the step-down reaction (Doughty and Diehn 1979, 1980, 1982, 1983, 1984; Doughty et al. 1980), the step-up reaction (Mikołajczyk 1984 a), the effects of pronase treatment on both photophobic responses (Walne et al. 1984), and the effects of low concentrations of external calcium on phototaxis (Meyer and Hildebrand 1988).

Mikołajczyk (1984 a) suggested that in *Euglena*, there may be an accessory chromophore for the step-up reaction, in addition to flavins in the flagellar swelling that are responsible for the step-down and the step-up responses. Such an accessory chromophore would probably be located in the flagellar membrane, close to the flagellar swelling (PFB). It has also been shown that *Astasia longa* exhibits a step-up photophobic reaction similar to the in *E. gracilis* (Suzaki and Williamson 1983; Mikołajczyk 1984 b).

On the basis of these data, Mikołajczyk and Kuźnicki (1984) speculated that the photopigment in the colorless, osmotropic *A. longa* and an accessory chromophore in the chloroplast-containing, photosynthetic *E. gracilis* would be similar and would represent the primary chromophore of a colorless phagotrophic ancestor of extant euglenoid flagellates.

During the last two decades, photophysiologists have paid much more attention to the step-down than to the step-up reaction in euglenoids. In that regard, Diehn (1985) remarked, "We have chosen to investigate the step-down photophobic response of *Euglena* because that response is the behavioral basis of photoaccumulation as well as phototaxis in the cell." We believe that many of the difficulties and controversies surrounding photophobic responses in *E. gracilis* are due, in large part, to the generally separate investigations of both reactions. In reviewing the literature, we have found sufficient data on the subject to carry out a comparative study and to propose a new hypothesis for a common sensing mechanism for the photophobic responses in *E. gracilis*.

Factors Determining the Threshold Values of the Step-up Photophobic Response

Since the beginning of quantitative studies on light-induced motor reactions of *Euglena gracilis*, it has been clearly understood that the threshold values of step-down and step-up responses are strongly influ-

http://rcin.org.pl

enced by different factors, mainly illumination and culture conditions (Diehn 1969 a, b; Diehn et al. 1975). More recent data have shown that (1) Streptomycin-bleached cells respond to the light at very low fluence rates and do not exhibit any step-down photophobic reaction (Checcucci et al. 1976). (2) In solutions of the detergent CTAB, similar results occur with green specimens: step-down responses disappear, while the threshold for the step-up response is lowered significantly (Mikołajczyk and Diehn 1978). (3) In log-phase cultures, no step-down responses are observed, whereas step-up reactions are manifested by all cells (Colombetti et al. 1981). (4) Cells transferred from culture media to a buffer solution containing Ca^{2+}, Mg^{2+} and K^{+} ions lose the step-down response for several hours (Doughty and Diehn 1979, 1980, 1983). The disappearance of the step-down response

Table 1

Factors that completely inhibit the step-down photophobic response in *E. gracilis* and their effects on the step-up photophobic response

Factors that inhibit the step-down photophobic reaction	Effect on step-up photophobic reaction	Data from:
White light at fluence rate over 200 W/m²	Increases the threshold value.	Diehn (1969 b)
KI	Fully preserved. Threshold value has not been checked.	Diehn and Kint (1970) Mikołajczyk and Diehn (1975)
Streptomycin	Enhanced substantially. Decreases the threshold value in bleached cells.	Checcucci et al. (1976) Ferrara and Banchetti (1976)
Cetyltrimethylammonium bromide (CTAB)	Fully preserved. Threshold value decreased.	Mikołajczyk and Diehn (1978)
Hepes-Pipes buffer (pH 7.1) with Mg-Ca-K ions	Substantial decrease in the threshold value during first 4 h after rinsing.	Doughty and Diehn (1979, 1983), Mikołajczyk and Pado (1981), Mikołajczyk (1984 a)
Growth in the dark	Substantial decrease in the threshold value in bleached cells.	Colombetti et al. (1981) Mikołajczyk (1984 a)
Log phase of growth in light-grown cultures	Quite conspicuous. Threshold value has not been checked.	Colombetti et al. (1981)
Pronase	Suppressed in 50% of the specimens after 4 h at the highest concentration.	Walne et al. (1984)

http://rcin.org.pl

is accompanied by a decrease in the threshold of the step-up response (Mikołajczyk and Pado 1981).

The available data on the complete inhibition of the step-down photophobic response in *E. gracilis*, in relation to the step-up photophobic reaction are summarized in Table 1.

It has been obvious for some time that in *E. gracilis* the threshold for the step-up reaction decreases when, for some reason, the step-down reaction is restricted or does not occur, regardless of the factors affecting it. As soon as the cells recover the ability to perform the step-down response, the threshold for the step-up reaction increases. This relationship was the basis for the assumption that in *E. gracilis* either two different primary chromophores or two different sensory transduction chains operate for the step-up and the step-down photoresponses.

Comparative studies on the threshold of the step-up responses in light- and dark-grown *E. gracilis* and in *E. gracilis* and *A. longa* after rinsing in buffer solution (Mikołajczyk 1984 a, b) have provided important information on the relationship between step-up and step--down reactions. At wavelengths of 350–480 nm, the step-up thresholds of the dark-grown cells were lowered by factors of 3–5 compared to green cells grown in cool-white light (1.0 W/m^2).

In addition to growth in darkness, illumination with an active wavelength strongly affected the step-up photoresponse threshold. In experiments on light-induced motor response, green cells grown under continuous white light were maintained under dim red light, and dark-grown cells were kept in darkness prior to experimentation, in order to obtain comparable data. Illumination of *Euglena* cells by white or monochromatic light in the wavelength range of 350–520 nm not only increased the step-up response threshold, but also increased individual variation. For that reason, in quantitative studies of the step-up reaction, cells were stimulated only once, then discarded. Even milliseconds of illumination with a fluence rate higher than 10 W/m^2 increased the threshold of the step-up photothobic response with repeated stimulation (Mikołajczyk 1984 a).

When samples of both light- and dark-grown *Euglena* were rinsed with Hepes-Pipes buffer, pH 7.1, containing 0.5 mM $CaCl_2$, 0.125 mM $MgCl_2$, 1.0 mM KCl_2, their step-up response thresholds were lowered markedly after 20–30 min: in green cells by a factor of 9 in the spectral range 350–400 nm, while in dark-grown cells by a factor of 10 at wavelengths 350 and 450 nm (Mikołajczyk 1984 a). Likewise, cells of *A. longa* became much more sensitive to light after rinsing with the same buffer. For example, a sudden increase of fluence rate, up to 13.4 kW/m^2,

http://rcin.org.pl

did not effect the step-up response, but after a buffer rinse, light of a fluence rate of only 0.154 kW/m^2 evoked the step-up reaction in 100% of the cells in the observed field, and at a fluence rate of 30 kW/m^2, responses were detected in 10% of the cells (Mikołajczyk 1984 b).

Consequently, if the same buffer effects a decrease in the fluence rate threshold for the step-up response in light-and dark-grown cells of *E. gracilis*, as well as in colorless *A. longa* that lacks a flagellar swelling, it suggests that the phenomenon does not depend upon flavins located in the flagellar swelling. In both species, the photoreceptor chromophores must be located somewhere in the plasmamembrane; however, the molecules responsible for the phenomenon may not be the same.

In establishing the action spectrum for the step-up response of *A. longa*, Suzaki and Williamson (1983) found the range (350–520 nm) to be identical to that for light-grown *E. gracilis*; however, the shape of the curve and the maximum response of *A. longa* (at 400 nm) are quite different from the action spectra for step-down and step-up reactions of *E. gracilis* (Checcucci et al. 1976; Barghigiani et al. 1979).

The existence of an "accessory" photoreceptor pigment in the flagellar membrane of *E. gracilis* and *A. longa*, in addition to flavins in the flagellar swelling, was proposed by Mikołajczyk (1984 a, b) and Mikołajczyk and Kuźnicki (1984). We agree that, in all likelihood, the membrane that covers flagella, reservoir and canal contains a complex of flavoproteins which, in addition, to their enzymatic function, absorb photons of a certain fluence rate. We have termed this system the "plasmamembrane photosensitive system" (PPS). When brought to an excited level, the flavoproteins of the PPS would initiate the photosensory chain for the step-up photophobic response in *E. gracilis* when the step-up threshold is drastically decreased. In *A. longa*, only the PPS system operates routinely and in this species includes the membrane covering the reservoir and flagellum. In contrast, in *Peranema trichophorum*, the entire cell surface is sensitive to the light, but with a strong anterior-posterior gradation of sensitivity (Mikołajczyk 1986). In fact, in most, if not in all euglenoids that have photoreception and photosensory transduction, those phenomena involve membrane-associated chromophores. In the sense that their occurrence is more "universal" they should probably be considered as the "primary" chromophores. Those photopigments that are located in more specialized structures of limited occurrence, such as the flagellar swelling, and that seem to serve more restricted functions should perhaps be designated as "accessory" chromophores.

http://rcin.org.pl

The Conduction-Resistance Properties of the Plasmamembrane and Their Role in Establishing the Threshold Value for the Step-up Photophobic Response

The hypotheses of different primary chromophores for the step-up and step-down photophobic response, and of dual transduction chains, are based on different sensitivities to a variety of factors. Some agents abolish the step-down reaction but have no effect on the step-up response (see Table 1). In our opinion, each example of selective inhibition of the step-down photophobic response can be better explained in ways other than have already been suggested.

For example, the hypothesis of two different primary chromophores and the supposition that flavins may not be the photoreceptor pigments for the step-up photophobic reaction of *E. gracilis*, originated on the basis of experiments with Kl (Diehn and Kint 1970; Mikołajczyk and Diehn 1975). From chemical experiments it was well known that KI quenches an excited state of flavins. In KI solutions, *E. gracilis* exhibits only the step-up photophobic response and photodispersion. Yet, KI could not selectively inhibit the step-down response as an effect of I^{-1} ions because (1) I^{-1} ions penetrate the plasmamembrane only with great difficulty, and (2) after intracellular injection they are very toxic. Thus, conjectures on the existence of two photoreceptor systems (Mikołajczyk and Diehn 1975) or two sensory transduction chains (Diehn 1979) may be premature.

Modification of the photobehavior of *E. gracilis* in KI solution is related only to the effects of K^+ ions and not at all to I^{-1} ions. As Doughty and Diehn (1979, 1983) and Doughty et al. (1980) showed, potassium ions alone, as well as together with Ca^{2+} ions in Hepes-Pipes adaptation buffer, strongly influenced the step-down response, including even its total disappearance. In buffer-rinsed cells the disappearance of the step-down response was simultaneous with a decrease in the threshold value for the step-up response (Mikołajczyk and Pado 1981; Mikołajczyk 1984 a). In the light of these data, it is clear that K^+ ions specifically changed the plasmamembrane covering the flagellum, canal and reservoir, thus contributing to the significant decrease in the threshold for the step-up photophobic response. When the threshold value dropped to 10^{-2}–10^{-3} W/m^2 for the step-up photophobic reaction, the possibility of te appearance of the step-down reaction was automatically excluded. Exactly te same results occurred in solutions of the anionic detergent CTAB (Mikołajczyk and Diehn 1978).

These data support our idea that all drug-induced effects which de-

http://rcin.org.pl

crease the step-up photophobic response threashold, concomitant with the disappearance of the step-down reaction, are limited to the plasmamembrane and do not involve changes in the chromophores of the flagellar swelling (PFB).

We propose that these large-scale changes in the threshold of the step-up photophobic response, even greater than for dark-grown or streptomycin-bleached cells, are brought about by changes in conduction-resistance properties of the membrane covering the flagellum, canal and reservoir. The molecular nature of such changes in the membrane of *Euglena* must still be determined, but the phenomenon is well known in cell electrophysiology.

We further propose that the plasmamembrane phototransduction processes occur via a "gate system" that may widen or narrow with changing external condition over time. For example, Mikołajczyk (1984 a) showed that after rinsing *E. gracilis* cells with Hepes-Pipes Mg-Ca-K buffer, the lowered threshold for the step-up response in light--grown (green) and dark-grown specimens appeared 20–30 min later and persisted for 1–4 h. Subsequently, the threshold value gradually increased and simultaneously, in the green cells, the step-down photophobic reaction reappeared gradually, up to its pre-rinsing level. The dark-grown flagellates behaved differently. At wavelengths of 350–510 nm, the threshold value for the step-up response did not return to its pre-rinsing level but rose more than 30 times higher than the controls. In some samples of dark-grown *E. gracilis*, te step-up threshold increased to the highest level immediately after rinsing, a result never observed in green cells (Mikołajczyk 1984 a).

The low threshold for step-up photophobic response (10^{-3} W/m^2) means that even if a small number of chromophore molecules in the flagellar swelling and plasmamembrane sites are exited by a low fluence rate, the stimulus affects the flagellar axoneme as a consequnence of high membrane conduction and amplification processes. At a high fluence rate (10^2 W/m^2), when the primary molecular events occur in the majority of photochromophore molecules, *E. gracilis* may not exhibit any motor reaction because of the high membrane resistance for the phototransduction processes.

Presumable Molecular Events in the Flagellar Swelling

The molecular events initiating the photophobic responses are completely unknown in any of the euglenoid flagellates. As yet, no flavoproteins have been isolated from *E. gracilis*, even though there are many

http://rcin.org.pl

reasons (Song 1983) to suppose that the chromophore in the flagellar swelling is retained with the protein. On the basis of the presumed interconnections between the flavin and protein moieties of the chromophores, we would now like to speculate on the first molecular events associated with the step-up and step-down photophobic reactions.

At moderate light intensities in the green flagellates, for example, the first step of a phototransduction process occurs in the flagellar swelling (PFB). The initial reactions for the step-up photophobic response are determined by the number of flavin molecules in the flagellar swelling that are excited by light.

While the step-up reaction and negative phototaxis occur in a wide range of fluence rates (i.e., from a light stimulus of about 10^{-3} W/m^2 up to a fluence rate that may injure or destroy the flagellates), the step--down reaction and positive phototaxis are phenomena of moderate light and occur in a relatively narrow range of temporary changes in fluence rate. As shown by Diehn (1969 b) and confirmed by most of the later research, both reactions appear in green specimens of *E. gracilis* at fluence rates below 200 W/m^2. After exposure to white light with fluence rates of such intensity, sudden dimming (e.g., to 1.0 W/m^2) evoked the step-down reaction in only a small percentage of cells in the probe. Upon lowering the illumination, the percentage of reacting organisms increased and reached the optimum expression of the step-down response at about 10 W/m^2. The lowest fluence rate capable of evoking the step-down photophobic response was of the order of 10^{-2} W/m^2 (Checcucci et al. 1974, Creutz and Diehn 1976). At these illuminations, exactly as in the highest illumination (close to 200 W/m^2), only individual specimens of *E. gracilis* exhibited the step-down reaction.

Experiments with individual *Euglena* cells (Barghigiani et al. 1979; Doughty and Diehn 1980) showed that upon their removal from a fluence rate of about 0.3×10^{-2} W/m^2, there was only a 10% probability of evoking the step-down response. From this lowest intensity up to about 10 W/m^2, the strength of expression of the step-down photophobic response increased linearly with the logarithm of the fluence rate. From about that point, the step-down reaction decreased linearly and completely disappeared, usually before the fluence rate reached 200 W/m^2.

Positive phototaxis in *E. gracilis* is exhibited in the same or rather more narrow range of light intensities, however never achieving close to the 100% specimen response as for the step-down reaction (Fig. 1). The bell-shaped form of the intensity-effect curves for green specimens growing in moderate light shows that the highest percentage of responses for positive phototaxis occurs between 1–10 W/m^2. These data for both

http://rcin.org.pl

light-induced motor responses provide some important information about how the underlying molecular mechanism probably works.

Foster and Smyth (1980) explained the bell-shaped form of the intensity-effect curves in positive phototaxis and the slope of the phototactic efficiency at a higher fluence rate (Fig. 1) as an effect of a de-

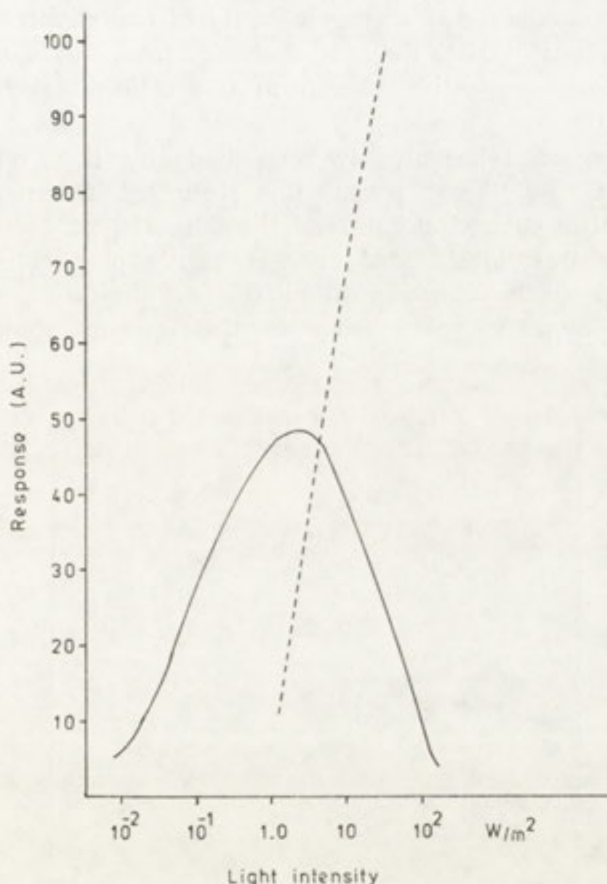


Fig. 1. Generalized intensity-effect curves of phototaxis evoked by white light in a population of green *Euglena gracilis*. Solid line: positive phototaxis; dashed line: negative phototaxis. Abscissa: fluence rate in W/m^2; ordinate: photomotile response in arbitrary units (A.U.)

crease in the availability of excitable chromophores with an increase in the light intensity. Colombetti and Petracchi (in press) argue that such a suggestion is in conflict with the simultaneous disappearance of positive phototaxis with the appearance of negative phototaxis. This relationship is well known in *E. gracilis* but is expressed much

http://rcin.org.pl

more strongly by the photophobic reactions (Fig. 2) than by such transient positive-negative phototaxis. For example, in the range of fluence rates from 10 W/m^2 up to about 200 W/m^2, the same linear relationship expresses intensity effect from nearly 100% to 0% for the step-down response and from 0% to 100% for the appearance of the step-up response. We believe that there is only one possible interpretation of these phenomena. In moderate light, the intensity of expression of the phobic responses is proportional to the rate of change from one form of a photoreceptor chromophore to the other form at a ratio directly proportional to changes in the fluence rate.

Such a suggestion has already been made by Kuźnicki (1988), and we would now like to pursue this argument. Green specimens of *E. gracilis* with a colored stigma and the normal (not decreasing or increasing) plasmamembrane conduction-resistance properties exhibit the step-down photophobic response at moderate light intensities. The response is in a dynamic relationship to the step-up photophobic res-

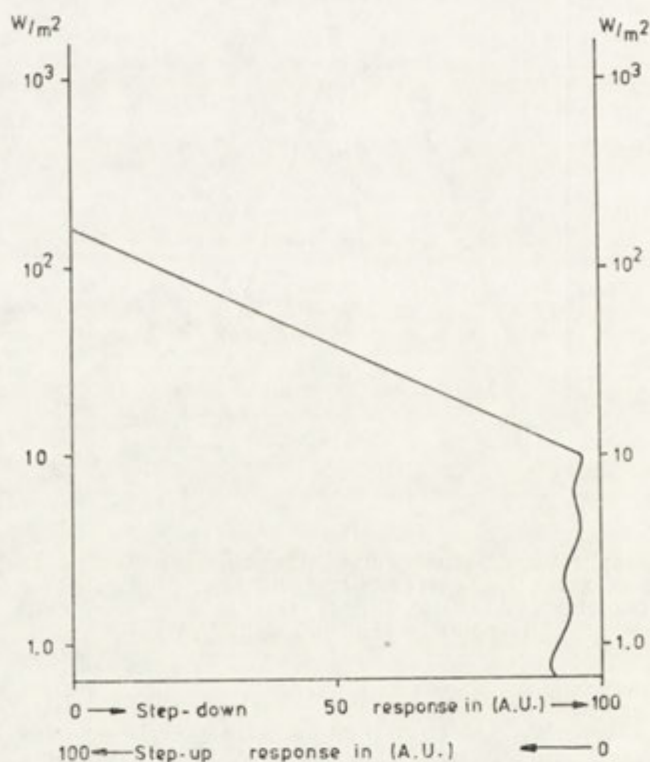


Fig. 2. Expression of photophobic response by green *Euglena gracilis* depends upon the intensity of white light

http://rcin.org.pl

ponse. This dynamic relationship is dependent upon the fluence rate (Fig. 2), and, in our opinion, reflects changes in the proportion of the two distinct signaling forms of flavoproteins in the flagellar swelling. It is generally believed that the initial steps in the transduction chain leading to the step-up response are determined by the number of chromophores that absorb photons. We assume that the fraction of the flavoproteins brought to an excited level when the flagellar swelling is illuminated undergoes, simultaneously, certain conformational changes. We have referred to such changes as "compact" or "unfolded" forms of the photoreceptor molecules in the flagellar swelling. The conformational changes might also be consistent with a liquid crystal structure of the flagellar swelling, but this untested possibility remains an open question.

The compact forms of flavoproteins are photochemically quiescent molecules, but conversion to the previously unfolded structure with the isoalloxazyne ring providing easy access for photons, generates the signal for the step-down photophobic response. Whereas the transition from the unfolded molecule to the compact one requires photon absorption, the conversion back to the native form occurs spontaneously, but the speed of conversion is strongly related to the fluence rate. The illumination of green *E. gracilis* cells by a fluence rate above 200 W/m^2 makes conversion impossible after a sudden decrease in fluence rate. Only prolonged stays in darkness or at low light intensities resorte the photoreceptory system of strongly illuminated flagellats. In moderate light intensities (1.0–10 W/m^2), a sudden decrease of fluence rate activates the conversion process in PFB flavoproteins.

The concept of compact and unfolded forms of photereceptor flavoproteins in the PFB is only a working hypothesis; however, such processes are well known in protein chemistry, and the idea of two distinct signaling forms fit very well with the majority of data on photobehavioral responses of *E. gracilis*.

The Role of the Stigma

That a stigma might act as a quarter-wave interference light reflector in unicellular eukaryotes has been suggested by Foster and Smyth (1980). In *E. gracilis*, however, the stigma acts just opposite to their suppostition. With an increase of stigma transmittance, there is a gradual disappearance of the step-down photophobic response and a simultaneous decrease in the step-up response threshold (Checcucci et al. 1976). This phenomenon occurs regardless of any factor that effects bleaching of cells (e.g., growth in darkness, streptomycin treatment).

http://rcin.org.pl

The relation between stigma transmittance and light-induced motor reactions of *E. gracilis* (stigma-less specimens show only the step-up photophobic response and are capable of negative phototaxis) led to the supposition of dual chromophores or dual sensory transduction chains for the photophobic reactions (Colombetti et al. 1982). Recently, Colombetti and Petracchi (in press) suggested that "formation of the stigma runs parallel to the synthesis of some other factors necessary to the expression of the step-down photophobic response." It is clear that the transmittance of the stigma modulates the number of photons absorbed by flavoproteins in the flagellar swelling at a given time. The threshold value for the light-grown cells with deeply colored stigma must be much higher than for the stigma-less, bleached cells, and that has been found in all cases. The cells are able to detect a temporal decrease in the fluence rate if their photoreceptor chromophores in the flagellar swelling are well protected by carotenoids in the deeply colored stigma. Reduction or lack of such protection is the major reason why dark-grown and streptomycin-bleached cells do not exhibit the step-down response.

Several authors have proposed that the stigma in *Euglena* does not play any direct role in photoreception (Colombetti et al. 1982; Song 1983; Colombetti and Petracchi i in press). Thus, it is, particularly confusing to designate both the flagellar swelling and the stigma as photoreceptors, and flavins and carotenids as the chromophares of photoreceptors. In aur conception, the stigma is a photoprotective structure, while carotenoids and other chromophores in the stigma are photoprotective molecules. This would be true for all euglenophyte species that contain a stigma and flagellar swelling.

Recognition of the stigma as a photoprotective organelle and of carotenoids as photoprotective molecules would explain their roles in the photophobic responses of *E. gracilis* but still provides no new insights into the mechanisms of photoorientation to the light source. Whether negative phototaxis in *E. gracilis* is brought about by repetitive step-up photophobic responses, and positive phototaxis by step-down reactions, or whether cells detect the direction of the light source by a dichroic orientation of flavins in the flagellar swelling (Häder 1987) remains an open question.

In the most recent studies on phototaxis in *Euglena*, Häder (1987) rejected the idea of a relationship between phobic responses and orientation to the light sources, but some close functional similarities are existing. In stigma-less specimens of *E. gracilis*, for example, the step-down photophobic reaction disappears, as des positive phototaxis. Streptomycin- or dark-bleached cells show only negative phototaxis and the step-up response (Häder et al., in press).

http://rcin.org.pl

Concluding Remarks

Colombetti et al. (1982) concluded that an understanding of the basic molecular mechanisms of photoreception could come from comprehension of the factors affecting the thresholds of the step-down and step-up reactions and of their mechanisms.

The basic task of this communication has been to show that the present state of knowledge on light-induced motor reactions of *E. gracilis* leads to the conclusion that there is an integrated molecular mechanism for photophobic responses. Thus, we propose that only one sensing mechanism for the step-up and step-down rections exists, despite the involvement of different chromophores in different parts of the cell.

On the basis of all available data on light-induced motor responses, we suggest that in *E. gracilis* the mechanism for the step-up response determines the expression of the step-down response, regardless of the agents that evoke changes in the sensitivities to light.

At very low and very high thresholds for the step-up photophobic response, the step-down responses are totally excluded: at the low, because the PFB flavoprotein systems are unable to detect the difference in light intensity; at the high threshold, a high fluence rate immobilizes the compact forms of the photoreceptor in the flagellar swelling.

The conclusions and some controversial suggestions that have been made in the present paper can serve, we believe, as an outline of a new methodological approach to studies of the light-induced motor responses of euglenoid flagellates. Many open questions remain, including the need for further clarification of phototransduction processes and the roles and precise internal structures of the flagellar swelling, the stigma, and the plasmamembrane photosensitive system, as well as their functional interrelationships in the photophobic responses of *E. gracilis*.

ACKNOWLEDGEMENTS

This work was supported in part by NSF Grant No. BSR 8508483 to P.L.W. and by Grant No. CPBP-04.01 to L.K.

REFERENCES

Barghigiani C., Colombetti G., Francinni B. and Lenci F. 1979: Photobehavior of *Euglena gracilis* — Action spectrum for the step-down photophobic response of individual cells. Photochem. Photobiol., 29, 1015–1019.

Checcucci A., Colombetti G., Ferrara R., Lenci F. 1974: Modellimolecolari per la fototassi in *Euglena gracilis*. Proc. Natl. Congr. Biophys. Cybernet., 3, 275–282.

http://rcin.org.pl

Checcucci A., Colombetti G., Ferrara R., Lenci F. 1976: Action spectra for photoaccumulation of green and colorless *Euglena*; evidence for identification of receptor pigments. Photochem. Photobiol., 23, 51–54.

Colombetti G., Lenci F. and Diehn B. 1982: Responses to photic, chemical and mechanical stimuli. In: The Biology of *Euglena*. Vol. 3, Physiology, (ed. D. E. Buetow), Academic Press, New York, 169–195.

Colombetti G., Lenci F., Passarelli V., Quaglia M. and Ronaldi-Fornaca C. 1981: Are there two separate photosensory transduction chains in *Euglana?* Progress in Protozoology. Abstract of papers submitted to Sixth Int. Congr. Protozool., Warszawa, Poland, July 5–11, 1981, 61.

Colombetti G. and Petrachi D. 1989: Photoresponse mechanisms in flagellated algae. C.R.C. Critical Reviews in Plant Science. C.R.C. Press, Boca Raton, Florida, (in press).

Creutz C. and Diehn B. 1976: Motor responses to polarized light and gravity sensing in *Euglena gracilis*. J. Protozool., 23, 552–556.

Diehn B. 1969 a: Phototactic response of *Euglena* to single and repetitive pulses of actinic light: orientation time and mechanism. Expl. Cell Res., 56, 375–381.

Diehn B. 1969 b: Action spectra of the phototactic responses in *Euglena*. Biochim. Biophys. Acta, 177, 136–143.

Diehn 1972: The receptor-effector system of phototaxis in *Euglena*. Acta Protozool., 11, 325–332.

Diehn B. 1979: Photic responses and sensory transduction in protists. In: Handbook of Sensory Physiology, (ed. H. Autrum), Springer Verlag, Berlin, New York, 7, 6A, 23–68.

Diehn B. 1980: Photomovement and photosensory transduction in microorganisms. Photochem. Photobiol., 31, 641–644.

Diehn B. 1985: Sensory transduction in *Euglena*. In: Sensory Perception and Transduction in Aneural Organisms. NATO ASI Series, Series A: Life Sciences Vol. 89, (eds. G. Colombetti, F. Lenci and P.-S. Song), 165–178.

Diehn B. and Kint B. 1970: The flavin nature of the photoreceptor pigment for phototaxis in *Euglena*. Physiol. Chem. Phys., 2, 483–488.

Diehn B., Fonseca J. R. and Jahn T. L. 1975: High speed cinemicrography of the direct photophobic response of *Euglena* and the mechanism of negative phototaxis. J. Protozool., 24, 492–494.

Doughty M. J. and Diehn B. 1979: Photosensory transduction in the flagellated alga, *Euglena gracilis*. I. Action of divalent cations, Ca^{2+} antagonists and Ca^{-} ionophore on motility and photobehavior. Biochim. Biophys. Acta, 588, 148–168.

Doughty M. J. and Diehn B. 1980: Flavins as photoreceptor pigments for behavioral responses in motile microorganisms, especially in the flagellated alga *Euglena* sp. A critique. Struct. Bond. 41, (ed. P. Hemmorich), Springer-Verlag, Berlin, New York, 45–70.

Doughty M. J. and Diehn B. 1982: Photosensory transduction in the flagellated alga, *Euglena gracilis*. III. Induction of Ca^{2+}-dependent responses by monovalent cation ionophores. Biochim. Biophys. Acta, 682, 32–43.

Doughty M. J. and Diehn B. 1983: Photosensory transduction in the flagellated alga, *Euglena gracilis*. IV. Long term effects of ions and pH on the expression of step-down photobehaviour. Arch. Microbiol., 134, 204–207.

Doughty M. J. and Diehn B. 1984: Anion sensitivity of motility and step-down photophobic responses of *Euglena gracilis*. Arch. Microbiol., 138, 329–332.

Doughty M. J., Grieser R. and Diehn B. 1980: Photosensory transduction in the flagellated alga, *Euglena gracilis*. 2. Evidence that blue-light effects alteration in Na^{+}-K^{+} permeability of the photoreceptor membrane. Biochim. Biophys. Acta, 601, 10–23.

Ferrara R. and Banchetti R. 1976: Effect of streptomycin on the structure and function of the photoreceptor apparatus of *Euglena gracilis*. J. Exp., Zool., 198, 393–402.

Foster K. and Smyth R. D. 1980: Light antennas in phototactic algae. Microbiol. Rev., 44, 572–630.

Häder D.-P. 1987: Polarotaxis, gravitaxis and vertical phototaxis in the green flagellate *Euglena gracilis*. Arch. Microbiol., 147, 179–183.

http://rcin.org.pl

Häder D.-P., Lebert M. and Dilena M. R. 1988: Effects of culture age and drugs on phototaxis in the green flagellate *Euglena gracilis* (in press).
Kuźnicki L. 1988: Mechanism of photophobic responses of *Euglena gracilis*; A hypothesis. In: Abstract Fourth Int. Congr. Cell Biol., Montreal, 379.
Meyer R. and Hildebrand E. 1988: Phototaxis of *Euglena gracilis* at low external calcium concentration. J. Photochem. Photobiol., B. Biol., 2, 443–453.
Mikołajczyk E. 1984 a: Photophobic responses in *Euglenina*: 1. Effects of excitation wavelength and external medium on the step-up response of light- and dark-grown *Euglena gracilis*. Acta Protozool., 23, 1–10.
Mikołajczyk E. 1984 b: Photophobic responses in *Euglenina*. 2. Sensitivity to light of the colorless flagellate *Astasia longa* in low and high viscosity medium. Acta Protozool., 23, 85–92.
Mikołajczyk E. 1986: Na/K transport and photosensitivity of the colorless flagellate *Peranema trichophorum* (*Euglenida*). Photochem. Photobiol., 43, 455–459.
Mikołajczyk E. and Diehn B. 1975: The effect of potassium iodide on photophobic responses in *Euglena*: Evidence for two photoreceptor pigments. Photochem. Photobiol., 22, 269–271.
Mikołajczyk E. and Diehn B. 1976: Light-induced body movement of *Euglena gracilis* coupled to flagellar photophobic response by mechanical stimulation. J. Protozool, 23, 144–147.
Mikołajczyk E. and Diehn B. 1978: Morphological alterations in *Euglena gracilis* induced by treatment with CTAB (Cetyltrimethylammonium bromide) and Triton X-100: correlations with effects on photophobic behavioral responses. J. Protozool., 25, 461–470.
Mikołajczyk E. and Kuźnicki L. 1984: Speculation on the origin of two photoreception systems in *Euglena*. Postępy Biol. Kom., 11, 411–413.
Mikołajczyk E. and Pado R. 1981: The kinetics of photobehavior modification upon change of the suspension medium of *Euglena gracilis*. Progress in Protozoology Abstr. Sixth Int. Congr. Protozool., Warszawa, Poland, July 5–11, 1981, 1.
Song P.-S. 1983: Protozoan and related photoreceptors: Molecular aspects. Ann. Rev. Biophys. Bioeng., 12, 35–68.
Suzaki T. and Williamson R. E. 1983: Photoresponse of a colorless euglenoid flagellate, *Astasia longa*. Plant Sci. Lett., 32, 101–107.
Walne P. L., Lenci F., Mikołajczyk E. and Colombetti G. 1984: Effect of pronase treatment on step-down and step-up photophobic responses in *Euglena gracilis*. Cell Biol. Int. Rep., 8, 1017–1027.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 231-237 (1989)

La Morphologie et Stomatogenèse de *Spathidium amphoriforme* (Greeff, 1888) (*Ciliophora: Kinetofragminophora*)

Janusz FYDA

Laboratoire d'Hydrobiologie, Institut de Biologie d'Environnment, Université Jagellone, Oleandry 2 a, 30-063 Kraków, Pologne

Received on 10th June, 1988, *revised on* 14th November, 1988

Synopsis. Ce travail présente les observations sur la morphologie et la stomatogenèse chez *Spathidium amphoriforme* (Greeff, 1888), isolé de la mousse. On donne la caractéristique biometrique des ciliés imprégnés au protargol. Le processus morphogenètique de notre espéce est comparé avec les autres descriptions de la morphogenèse chez *Spathidium.*

Plusieurs chercheurs: Wenzel (1953), Gellert (1955), Dragesco (1966), Fryd-Versavel et al. (1975), Foissner (1984), Dragesco et Dragesco-Kernéis (1986) ont étudié la morphologie de *Spathidium amphoriforme* "in vivo" ou bien sur les préparations argentiques.

Ce travail présente la description d'un cilié qui a été determiné comme *S. amphoriforme.* Plusieurs différences, concernantes la morphologie et la stomatogenèse chez cette espèce ont été démontrées par rapport aux travaux récents.

Matériel et Techniques

Spathidium amphoriforme a été trouvé dans la mousse provenant de Beskid Niski (sud de la Pologne). Les ciliés conservés dans la solution de Pringsheim étaient nourris avec *Colpidium campylum* Stokes, 1886. La morphologie et la stomatogenèse ont été étudiées "in vivo" ainsi que sur les préparations imprégnées au protargol d'après la méthode de Wilbert (1975), avec $HgCl_2$ saturé camme fixateur.

Les dessins ont été faits à l'aide de "camera lucida". Les données numeriques concernent des speciments colorés.

http://rcin.org.pl

Morphologie et Stomatogenèse

La cellule ressemble à l'amphore avec la spatule très évasée. La taille est assez grande, mais très variable. La longueur varie entre 140–203 μm et la largeur entre 55–106 μm. Le nombre des cinéties somatiques est aussi très variable: 39 à 51. (N=25). La "structure en brosse" consiste en trois courtes rangées de cinétosomes doubles. Les extrusomes ont en moyenne 6.6 μm de long. Les données biométriques sont présentés sur le Tableau 1. Le macronoyau en forme d'un long ruban est enroulé. Il y a plusieurs micronoyaux sphéroïdes. Une vacuole contractile est située au bout du corps.

Tableau 1

Données morphométriques de *Spathidium amphoriforme* (Greeff, 1888)

Caractère	$\bar{X}$	M	ET	CV	MIN	MAX	N
Longueur	170.23	170.04	16.80	0.10	140	203	25
Largeur maximale	79.00	81.12	12.22	0.15	55	106	25
Largeur minimale	40.37	39.00	5.86	0.15	31	55	25
Longuer du bourrelet buccal	59.65	60.84	6.13	0.10	47	73	25
Nombre des cinéties	44.00	43.00	3.18	0.07	39	51	16
Nombre des cinétosomes dans une cinétie	74.00	71.50	13.49	0.18	61	92	4
Nombre des cinéties dans la brosse	3.00	3.00	0.00	0.00	3	3	14
Nombre des pairs des cinétosomes dans la deuxième cinétie de la brosse	30.63	20.05	6.09	0.20	19	40	8
Longuer des extrusomes	6.64	6.82	1.15	0.17	4	9	24

Troutes les mesures en μm. CV — coefficient de variation, ET — écart type, M — médiane, MAX — maximum, MIN — minimum, $\bar{X}$ — moyenne arithmétique, N — nombre de spécimen.

La stomatogenèse chez *S. amphoriforme* commence au niveau de l'équateur par la multiplication des cinétosomes dans les cinéties dont les bouts antérieurs forment la brosse. Puis, la multiplication des cinétosomes s'étend sur les autres cinéties. Les cinétosomes proliferent parallèlements aux cinéties (Fig. 1 a, b; Pl. I 1). A l'étape suivant les cinéties se rompent au-dessus de la région de prolifération. Ensuite, les fragments antérieurs de chaque cinétie de l'opisthe se courbent (Fig. 1 c. d; Pl. I 2), jusqu'ils atteignent la cinétie précedante (Fig. 1 e; Pl. I 3). Chez l'individu adulte ces fragments, se courbent presque à 90° en formant une ligne autour de la bouche. Après la division les jeunes individus sont petits et arrondis.

http://rcin.org.pl

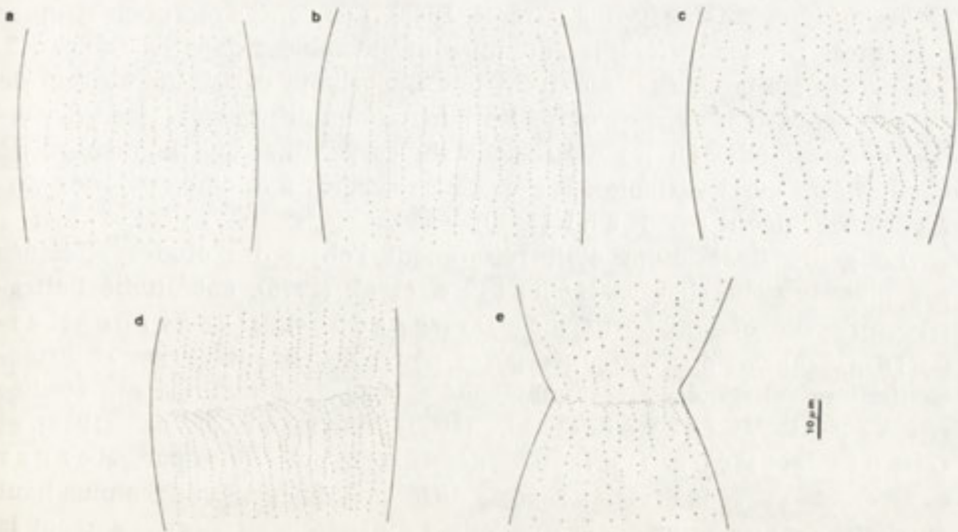


Fig. 1. Les stades successif de stomatogènese chez *Spathidium amphoriforme*: a–b — multiplication des cinétosomes dans les parties équatoriales des cinéties. Les cinétosomes proliferent parallèlements aux cinéties, c — rupture des cinéties au-dessus de la région de prolifération, d–e — infléchissement des cinéties. Les fragments antérieurs de chaque cinétie de l'opisthe se courbent, jusqu'ils atteingnent la cinétie précedante

Tableau 2

Données biométriques de *Spathidium amphoriforme* d'après divers auteurs

Auteur	Longueur en μm	Largeur en μm	No. des cinéteis
Kahl (1930)	120–200	—	20
	200–300[1]	—	50
	200–250[2]	—	—
Wenzel (1953)	110–180	—	—
Gellert (1955)	90–100[1]	—	40
Dragesco (1966)	120–260	—	40–48
Fryd-Versavel et al. (1975)	50–210*	30–80	40–50
Foissner (1984)	78–132*	31–56	24–38
Dragesco et Dragesco-Kernéis (1986)	50–250	30–80	—
Fyda	140–203*	55–106	39–51

[1] *S. amphoriforme* var. *securiforme* Kahl, 1930; [2] *S. amphoriforme* var. *rectitoratum* Kahl, 1930; * On a été mesuré les ciliés fixés et colorés.

http://rcin.org.pl

Discussion

Il y a plusieurs déscriptions des ciliés, qui correspondent à la diagnose de *Spathidium amphoriforme* (Greeff, 1888). Les donées morphometriques présentées par les différents auteurs sont assemblées dans le Tableau 2. Les caractères mesurés démontrent une importante variabilité au sein de chaque population ainsi qu'entre les populations différentes. Chez *S. amphoriforme* étudié ici les dimensions du corps ainsi que le nombre de cinèties sont aussi variables. Il s'approche surtout à *S. amphoriforme* var. *securiforme* décrit par Kahl (1930).

Le genre *Spathidium* était récemment l'objet des études effectués par plusieurs chercheurs. Bohatier et al. (1978), ont étudié l'ultrastructure de *S. amphoriforme*. Dragesco et Dragesco-Kernéis (1979) ont décrit deux espèces nouvelles: *S. muscorum* et *Protospathidium muscicola*. La stomatogenèse chez *Spathidium* a été étudiée par: Fryd-Versavel et al. (1975). Berger et al. (1983) et Czapik et Fyda (1987). La déscription présentée par Berger et al. (1983) est sous certains aspects différente des celles citées plus haut ainsi que de mes propres observations. La différence concerne sortout la forme de "primordia of perioral kinety". D'après Berger et al. (1983), ce sont les fragments des cinéties, où les cinétosomes prolifèrent perpen-

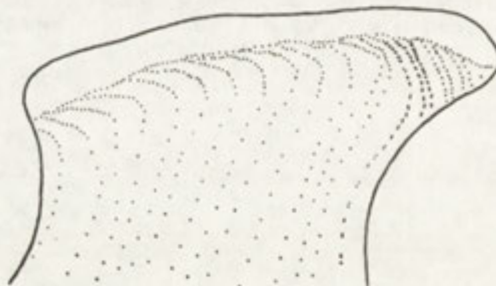


Fig. 2. Partie antérieure du corps — le côté gauche

diculairement aux cinéties, formant une rangée double. Sur les dessins des premières étapes de la morphogènese (Berger et al. 1983, Fig. 20–27), chaque primordium est représenté comme deux rangées cinétosomiennes. Ces soi-disants "primordia of perioral kinety" pendant la morphogenése se courbent jusqu'ils atteignent la cinétie précedante; de cette façon ils forment la cinétie circumorale de l'opisthe composée de deux rangées des cinétosomes (Berger et al. 1983, Fig. 25–28). Pourtant en examinant les préparations argentiques j'ai constaté que la multiplication des cinétosomes se produit parallèlement aux cinéties de l'opisthe (Fig.

http://rcin.org.pl

1 a, b; Pl. I 1). Chaque primordium a l'air d'une seule rangée des cinétosomes serrés. La bouche d'un adulte est entourée par une rangée des cinétosomes (Fig. 2; Pl. II 4, 5). Bohatier et al. (1978) ont démontré, qu'en réalité ce sont des paires de cinétosomes (un cilié, un autre non cilié), mais cette structure n'est pas visible au niveau optique.

En s'appuyant sur des nombreuses données morphologiques et morphométriques, Foissner (1984) a proposé une nouvelle classification de *Spathidiidae*. Il a décrit les nouveaux genres en vertu de la structure de la cinétie circumorale: "Hier werden mit Hilfe der Infraciliatur 2 weitere "gute" Genera errichtet und die Speciesdiagnosen vieler Arten bedeutend präzisiert" (Foissner, 1984). Le genre *Spathidium* est caractérisé d'après Foissner (1984) par une ciliature périorale simple. Dans la région orale les cinéties somatiques sont courbées et leurs extrémités s'approchent tout près l'un de l'autre; cela donne l'impression (surtout sur le côté gauche) d'une rangée cinétosomienne continue entourant la bouche (Fig. 3 b). Chez le genre *Epispathidium* la ciliature

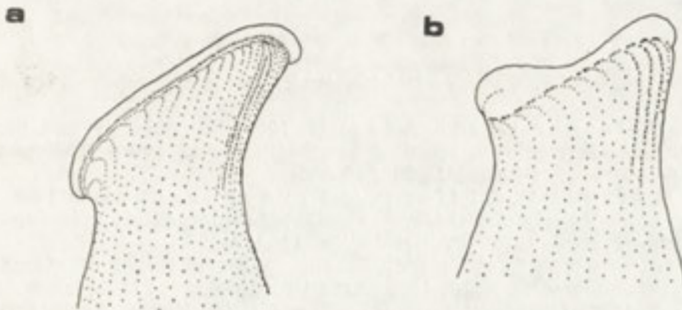


Fig. 3. a — Ciliature oral chez *Epispathidium amphoriforme* selon l'image de Foissner (1984, Fig. 42 f, pp. 85), b — Ciliature oral chez *Spathidium spathula* selon l'image de Foissner (1984, Fig. 34 r, pp. 69)

périorale est différente. Ce qui distingue ce genre nouveau, selon Foissner (1984), c'est présence d'une rangée cinétosomienne tout à fait indépendante (séparée) des cinéties somatiques. Cette rangée est parallèla à la ligne formée par les extremités des cinéties somatiques incour vées. "Spathidiidae, deren circumorale Kinete vollständig von den Somakineten getrennt ist. Anteriore Enden der Somakineten mit deutlich verdichteter Ciliatur, verlaufen wegen ihrer starken Abbregung vorne annähernd parallel zur circumoralen Kinete und weisen links nach ventral, rechts nach dorsal" (Foissner 1984, Fig. 33 k–l).

Spathidium amphoriforme est placé par Foissner (1984) dans le nouveau genre *Epispathidium*. Sur son dessin la ciliature périorale de *S. amphoriforme* est répresenté par deux lignes cinétosomiennes parallè-

http://rcin.org.pl

les et bien séparées, entourant la bouche. La première (cinétie circumorale) forme une ligne continue composée des paires des cinétosomes. La deuxième est formée par les extrémités incourvées des cinéties somatiques (Fig. 3 a).

S. amphoriforme étudié ici, se distingue bien de l'image donnée par Foissner (1984) en ce qui concerne la ciliature périorale. On ne voit ici qu'une seule ligne cinétosomienne formée par les fragments antérieurs courbés des cinéties somatiques (Fig. 2).

Si on compare les diagnoses des genres nouveaux *Epispathidium* et *Spathidium* ainsi que les images (Fig. 3 a, b) données par Foissner (1984), on voit clairement que notre espèce (Fig. 2) s'approche plutôt au genre *Spathidium* (Fig. 3 b), contrairement à cet auteur, qui le place dans *Epispathidium* (Fig. 3 a). Cela conduit à la conclusion suivante: ou bien notre espèce et celle étudiée par Foissner (1984) sont deux espèces différentes, qui se ressemblent par la forme générale du corps, mais qui se distinguent par leur ciliature périorale, ou bien le caractère proposé par Foissner (1984) est trop douteux pour créer les genres nouveaux.

BIBLIOGRAPHIE

Berger H., Foissner W. and Adam H. 1983: Morphology and morphogenesis of *Fuscheria terricola* n. sp. and *Spathidium muscorum* (*Ciliophora*: *Kinetofragminophora*). J. Protozool., 30, 529–535.

Bohatier J., Iftode F., Didier P. et Fryd-Versavel G. 1978: Sur l'ultrastructure des genres *Spathidium* et *Bryophyllum*, ciliés *Kinetophragmophora* (de Puytorac et al, 1974). Protistologica, 14, 189–200.

Czapik A. et Fyda J. 1987: L'étude sur la morphologie et stomatogenèse de *Spathidium muscicola* Kahl, 1930. Acta Protozool. 26, 291–294.

Dragesco J. 1966: Observations sur quelques ciliés libres. Arch. Protistenk. 109, 155–206.

Dragesco J., Dragesco-Kernéis A. 1979: Ciliés muscicoles nouveaux ou peu connus. Acta Protozool. 18, 401–416.

Dragesco J. et Dragesco-Kernéis A. 1986: Ciliés libres de l'Afrique intertropicale. Introduction à la connaissance et à l'étude des Ciliés. Faune Tropicale 26, L'Orstom, Paris, 153.

Foissner W. 1984: Infraciliatur, Silberliniensystem und Biometrie einiger neuer und wenig bekannter terrestrischer, limnischer und mariner *Ciliaten* (*Protozoa*: *Ciliophora*) aus der Klassen Kinetofragminophora, Colpodea und Polyhymenophora. Staphia, 12, 1–165.

Fryd-Versavel G., Iftode F., Dragesco J. 1975: Contribution a la connaisance de quelques ciliés gymnostomes. II. Prostomiens, Pleurostomiens: morphologie, stomatogenèse. Protistologica 11, 509–530.

Gellert J. 1955: Die Ciliaten der unter der Flechte *Parmelia saxatilis* Mass. gebildeten Humus. Acta. Biol. Acad. Sc. Hung. 6, 77–111.

Kahl A. 1930: Urtiere oder *Protozoa* I: Wimpertiere oder *Ciliata* (*Infusoria*), eine Bearbeitung der freilebender und ectocommensalen Infusorien der Erde, unter Auschluss der marinen Tintinnidae. In: Die Tirwelt Deutschlands, (ed. F. Dahl), G. Fischer, Jena, Teil 18.

Wenzel F. 1953: Die Ciliaten der Moosrasen trockner Standorte. Arch. Protistenk. 99, 70–141.

Wilbert N. 1975: Eine verbesserte Technik der Protargolimprägnation für Ciliaten. Mikrokosmos, 6, 171–179.

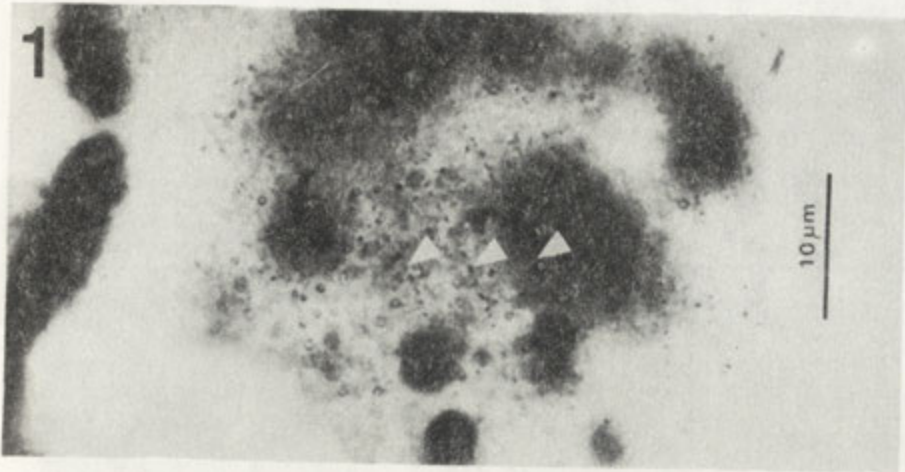
http://rcin.org.pl

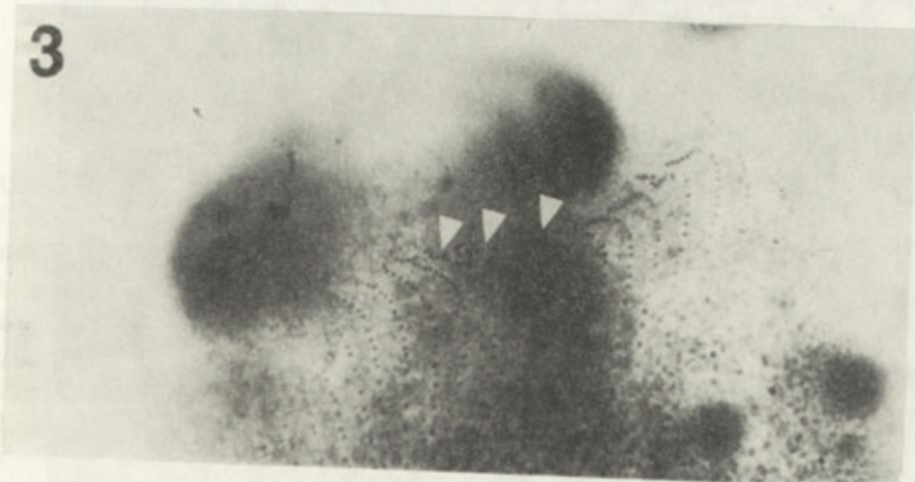
EXPLICATION DES PLANCHES I–II

1: Stomatogenèse chez *Spathidium amphoriforme*. Multiplication des cinétosomes dans la partie équatoriale
2: Rupture des cinéties
3: Infléchissement des cinéties
4: Partie antérieure du corps — le côté gauche
5: Partie antérieure du corps — le côté droit

http://rcin.org.pl

http://rcin.org.pl



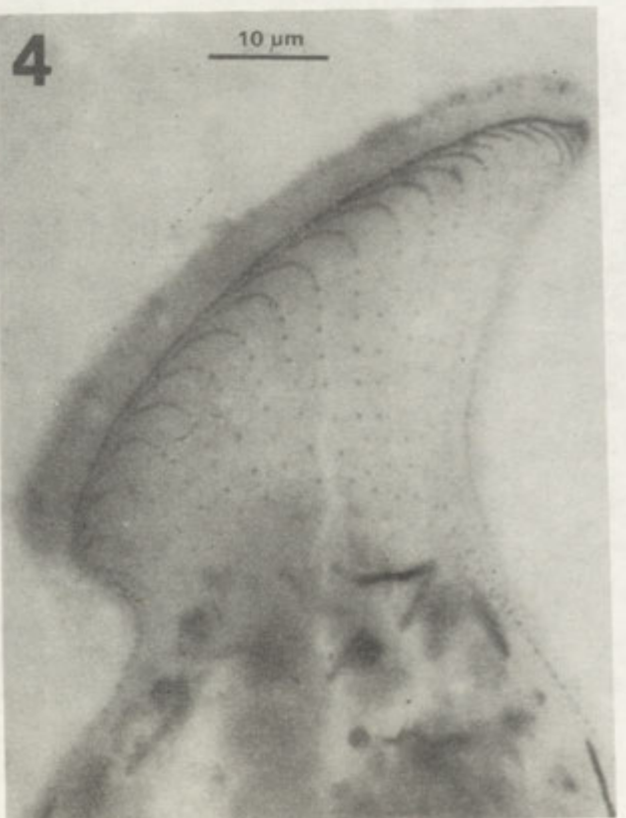


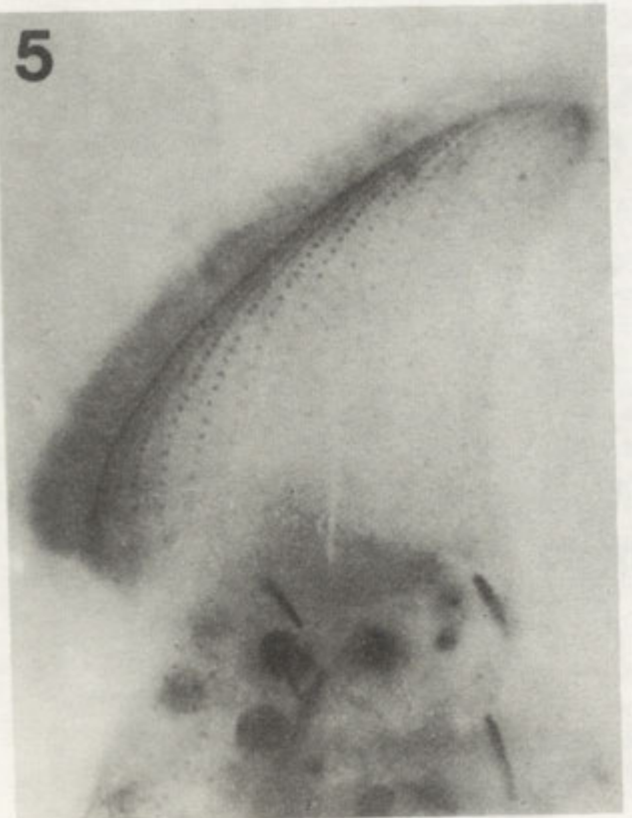
J. Fyda

auctor phot.

http://rcin.org.pl

PLATE II





J. Fyda

auctor phot.

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 239-244 (1989)

Light Microscopical Study of the Conjugation Process in *Opisthonecta henneguyi* Fauré-Fremiet (*Ciliophora: Peritrichida*)

A. SOLA, A. GUINEA and D. FERNANDEZ-GALIANO

Departamento de Microbiologia, Facultad de Biologia, Universidad Complutense, 28040 Madrid, Spain

Received on 18th July, 1988, *revised on* 12nd October, 1988

Synopsis. The conjugation process in *Opisthonecta henneguyi* begins with an asymetric division which originates a macroconjugant and a microconjugant cell. The complete microconjugant cell penetrates into the macroconjugant cell through the scopula, located at the aboral pole. The micronucleus of the microconjugant undergoes a preliminary division, and afterwards all the micronuclei of both conjugants undergo the first pregamic division, but only two of the nuclei of the microconjugant and one micronucleus of the macroconjugant undergo the second pregamic division. The synkarion is formed in the macroconjugant cell oral pole by fusion of the migratory pronucleus of the microconjugant with the stationary pronucleus of the macroconjugant. The synkarion divides thrice, yielding eight nuclei; one of them will be the micronucleus and the other seven become Anlagen or macronuclear primordia. Successive segregatinns of these Anlagen originate seven vegetative cells each one including one micronucleus and one macronucleus. The buccal and the trochal infraciliature of the macroconjugant do not undergo any modification during the conjugation process.

The conjugation process of peritrichous ciliates has received scarce attention (Dass 1953, 1954 a, b, Finley 1943, 1952, Maupas 1889, Mügge 1957, Raikov 1972, Rosenberg 1940, Seshacher and Dass 1951, Summers 1938) and little information is available at present. The morphology and division of species from free-motile sessiline peritrichous families (*Opisthonectidae* Foissner, 1975 and *Astylozoidae* Foissner, 1977) have been described (Bradbury 1965, Foissner 1975, 1977, Guinea et al. 1986, Lom 1964, Sola et al. 1985), but only one paper (Rosenberg 1940) reports the conjugation process of *Opisthonecta henneguyi*.

http://rcin.org.pl

In this study, the nuclear and the cortical events, especially those related to the buccal and the trochal infraciliature, originated during the conjugation process of this ciliate are reported, and some differences in respect to results of Rosenberg (1940) are described.

Material and Methods

Opisthonecta henneguyi was isolated from water collected at "La Dehesa de la Villa" (Madrid, Spain) and cultivated in lettuce-infusion. The specimens were impregnated using the Fernández-Galiano's silver carbonate method (1976).

Results

After inoculation of the cysts in fresh culture medium the cells divide asexually during the first 48 h, and about 48 to 72 h after the excystment the conjugation process starts. This process is detected by the presence of macro and microconjugant cells in the culture medium.

The duration of this process starting from the appearance of the conjugants in the culture until the great majority become vegetative cells, is about 48 h at room temperature (20°C).

The complete conjugation process may be divided in eight phases:

Preconjugation phase. This phase consists of one asymmetric division of the vegetative cell that results in two different conjugants (anisogamonts): a macroconjugant and a microconjugant (Pl. I 1). The morphology and way of swimming of the macronjugant is similar to that of the vegetative cell. The microconjugant is smaller and moves faster than the macroconjugant. The infraciliature of both conjugant cells, as viewed in the light microscopy, is similar to the parental cell (Pl. I 1).

Contact phase. The microconjugant cell swims in the culture medium very rapidly "in search of" a macroconjugant cell. The conjugation process "sensu stricto" begins with the contact between a microconjugant and a macroconjugant by means of their respective aboral poles (Pl. I 2). The conjugation pairs maintains the macroconjugant swimming behaviour or, in other words, the macroconjugant pushes the microconjugant. In this phase, the buccal and the trochal infraciliature of both conjugants (Pl. I 3) are not modified.

Penetration phase. Later, the complete microconjugant cell penetrates into the macroconjugant cell until the former rests at the aboral zone of the macroconjugant. In this zone, the cell membrane of the microconjugant can still be observed (Pl. I 4).

http://rcin.org.pl

Afterwards, the microconjugant cell membrane disappears (Pl. I 5) and its micronucleus undergoes a first mitotic division called by the authors "preliminary division" (Raikov 1972), giving rise to two micronuclei which can be observed in the aboral region of the macroconjugant cell (Pl. I 6).

Pregamic divisions. After the preliminary division of the micronucleus of the microconjugant, the first pregamic division of the micronuclei occurs in both conjugants. Thus, two haploid nuclei in the macroconjugant and four haploid nuclei in the microconjugant originate (Pl. II 7).

The second pregamic divisons occurs only in one of two haploid nuclei of the macroconjugant and in two of the four haploid nuclei of the microconjugant. The macronuclei of both conjugants degenerate (Pl. II 8).

During these pregamic divisions the macroconjugant nuclei are located at the oral pole while those of the microconjugant remain at the aboral pole.

Synkarion formation. In both microconjugant and macroconjugant only one nucleus remains intact which will become the pronucleus. The other ones disappear.

The microconjugant pronucleus migrates towards the oral pole of the cell, where it fuses with the macroconjugant pronucleus to produce the

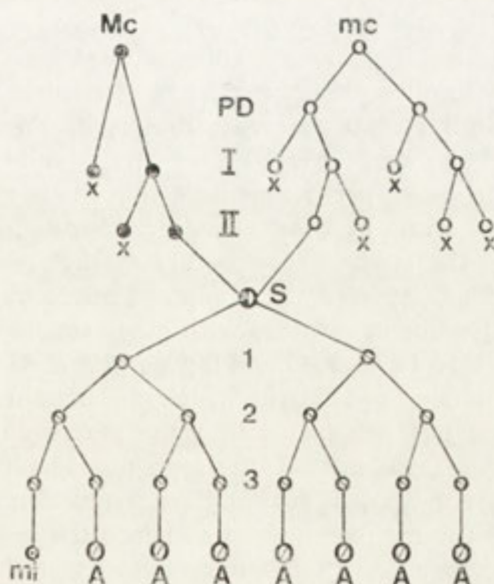


Fig. 1. Scheme of the nuclear behaviour in the conjugation process of *Opisthonecta henneguyi*. PD — preliminary division; I, II — pregamic divisions; 1, 2, 3 — postzygotic divisions; X — degenerating nuclei

http://rcin.org.pl

synkarion. At this stage of conjugation, the degenerating macronuclei show a clew-like structure, which preceeds the macronuclear fragmentation (Pl. II 9).

Postzygotic divisions. The synkarion divides thrice yielding eight nuclei, which initially cannot be differentiated from the macronuclear fragmentation products (Pl. II 10).

Segregation divisions. One of the eight nuclei will be the micronucleus while the other seven become Anlagen (Pl. II 11). These macronuclear Anlagen, during successive bipartition processes of the postconjugant cell, are segregated by all possible ways to form seven daugther cells, each one with one macronucleus and one micronucleus (Pl. II 12).

All this process is summarized in the scheme of Fig. 1.

During the postconjugation divisions, the stomatogenic process and the trochal band division are like those that occur normally in the vegetative cell division (Sola et al. 1985).

Discussion

After applying our impregnation method, we have obtained some new data about the conjugation process in *Opisthonecta henneguyi*. We have observed that the preconjugation division shows similar characteristics to those of the division of the vegetative cell (Sola et al. 1985). This division results in two free moving conjugants, which differs with that has been described in other sessile peritrichs studied in which the macroconjugant cell is stalked (Dass 1953, 1954 a, b, Finley 1943, 1952, Maupas 1889, Mügge 1957, Seshachar and Dass 1951). As a matter of fact, in other peritrichs the contact between the conjugants is somewhat lateral while in *Opisthonecta henneguyi*, as described Rosenberg (1940), the contact zone occurs at their respective scopulas.

In addition, in our species the complete microconjugant penetrates into the macroconjugant, in contrast with other sessile peritrichs (Dass 1953, 1954 a, b, Finley 1943, Mügge 1957, Seshachar and Dass 1951, Summers 1938) in which the membrane of the microconjugant cell does not penetrates into the macroconjugant. We agree with Bradbury (1965) in that the long aboral fibers of the trochal band, which do not disappear during the conjugation process, facilitate the penetration of the microconjugant cell into the macroconjugant.

The preliminary division of the micronucleus of the microconjugant reported previously in sessile peritrich ciliates (Raikov 1972) is confirmed by our study in *Opisthonecta henneguyi*. This preliminary division does not exist in mobiline peritrich ciliates (Raikov 1972).

http://rcin.org.pl

Our results concerning the number of nuclei that undergo a second pregamic division are differents than those reported for sessile peritrichs studied previously (Dass 1953, 1954 a, b, Finley 1943, 1952, Maupass 1889, Mügge 1957, Rosenberg 1940, Seshachar and Dass 1951 Summers 1938). In *Opisthonecta henneguyi* we have observed that one of the two macroconjugant micronuclei and two of the four microconjugant micronuclei undergo a second pregamic division. We have not observed the third pregamic division reported in *Vorticella* (Finley 1943, Maupas 1889, Mügge 1957, Seshachar and Dass 1951), *Carchesium* (Dass 1954 a, Maupas 1889) and *Zoothamnium* (Summers 1938). In *Opisthonecta henneguyi*, Rosenberg (1940) did not observe this third division either but he considered that it may have existed.

According to our results on our strain of *Opisthonecta henneguyi*, the synkarion is formed at the oral pole of the macroconjugant cell, in contrast with what has been reported by Rosenberg (1940). Then, in our opinion, the microconjugant pronucleus can be considered a migratory or male pronucleus while the macroconjugant pronucleus is the stationary or female pronucleus.

After the three postzygotic divisions, the seven macronuclear Anlagen are segregated by different ways during cell divisions. In these cell divisions, the behaviour of the buccal and the trochal infraciliatures is similar to that occurring during division of the vegetative cell (Sola et al. 1985).

In *Opisthonecta henneguyi*, as in some more evolved peritrichs, the third pregamic division does not exist, and in addition, in this species there is a reduction in the number of nuclei which undergo a second pregamic division. Consequently, in our opinion, from an evolutionary point of view, the conjugation process of *Opisthonecta henneguyi* should be considered more evolved than the processes described in other sessiline peritrichous ciliates.

REFERENCES

Bradbury P. C. 1965: The infraciliature and argyrome of *Opisthonecta henneguyi* Fauré-Fremiet. J. Protozool., 12, 345–363.

Dass C. M. S. 1953: Studies on the nuclear apparatus of peritrichous ciliates. I. The nuclear apparatus of *Epistylis articulata* From. Proc. Nat. Inst. Sci., India, 19, 389–404.

Dass C. M. S. 1954 a: Studies on the nuclear apparatus of peritrichous ciliates. II. The nuclear apparatus of *Carchesium spectabile* Ehrbg. Proc. Nat. Inst. Sci., India, 20, 174–186.

Dass C. M. S. 1954 b: Studies on the nuclear apparatus of peritrichous ciliates. III. The nuclear apparatus of *Epistylis sp.* Proc. Nat. Inst. Sci., India, 20, 703–715.

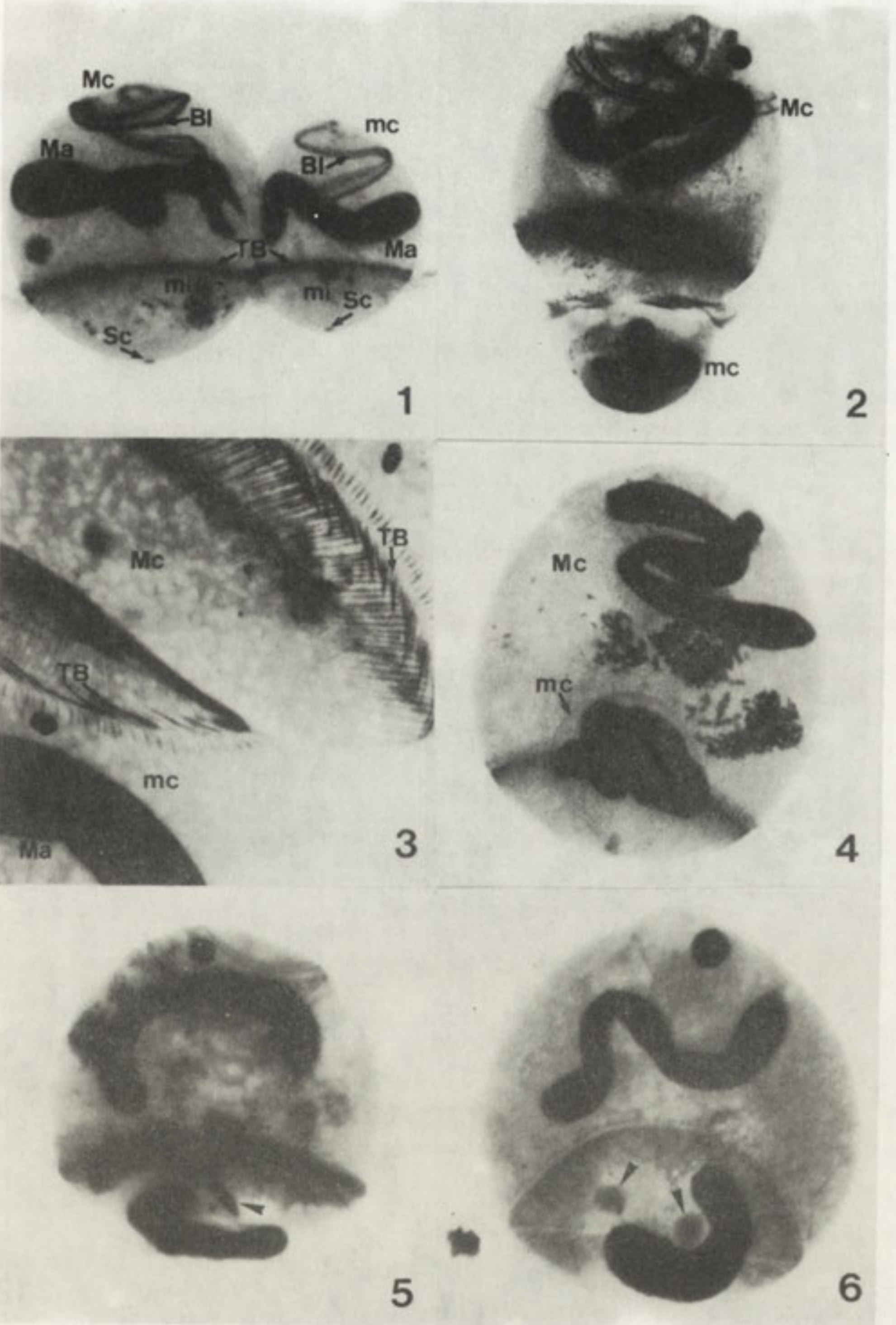
http://rcin.org.pl

Fernández-Galiano D. 1976: Silver impregnation of ciliated protozoa: procedure yielding good results with the pyridinated silver carbonate method. Trans. Am. Microsc. Soc., 95. 557–560.
Finley H. E. 1943: The conjugation of *Vorticella microstoma*. Trans. Am. Microsc. Soc., 62, 97–121.
Finley H. E. 1952: Sexual differentiation in peritrichous ciliates. J. Morphol., 91, 569–605.
Foissner W. 1975: *Opisthonectidae* (*Ciliata, Peritrichida*) nov. fam. und Revision der Genera *Telotrochidum* (Kent) und *Opisthonecta* (Fauré-Fremiet). Protistologica, 11, 395–410.
Foissner W. 1977: Revision der Genera *Astylozoon* (Engelmann) und *Hastatella* (Erlanger) (*Ciliata, Natantina*). Protistologica, 13, 353–379.
Guinea A., Gil R. and Fernández-Galiano D. 1986: Ultrastructure de la frange aborale d'*Opisthonecta henneguyi* Fauré-Fremiet, 1906 (*Ciliophora, Peritrichida*). Acta Protozool., 25, 15–22.
Lom J. 1964: The morphology and morphogenesis of the buccal ciliary organelles in some peritrichous ciliates. Arch. Protistenkd., 107, 131–162.
Maupas E. 1889: La rajeunissement karyogamique chez les ciliés. Arch. Zool. Exp. Gén., sér. 2, 7, 149–517.
Mügge E. 1957: Die Konjugation von *Vorticella campanula* (Ehrbg). Arch. Protistenkd., 102, 165–208.
Raikov I. B. 1972: Nuclear phenomena during the conjugation and autogamy on ciliates. In: Research in Protozoology, Vol. 4, (ed. T. T. Chen), Pergamon Press, Oxford.
Rosenberg L. E. 1940: Conjugation in *Opisthonecta henneguyi* a free swimming vorticellid. Proc. Am. Philos., 82, 437–446.
Seshachar B. R. and Dass C. M. S. 1951: The macronucleus of *Vorticella convallaria* (linn.) during the conjugation. J. Morphol., 89, 189–197.
Sola A., Guinea A. and Fernández-Galiano D. 1985: Etude du processus de bipartition chez *Opisthonecta henneguyi* Fauré-Fremiet, 1906 (*Ciliophora, Peritrichida*). Protistologica, 21, 333–338.
Summers F. M. 1938: Some aspects of normal development in the colonial ciliate *Zoothamnium alternans*. Biol. Bull., 74, 117-129.

EXPLANATION OF PLATE I–II

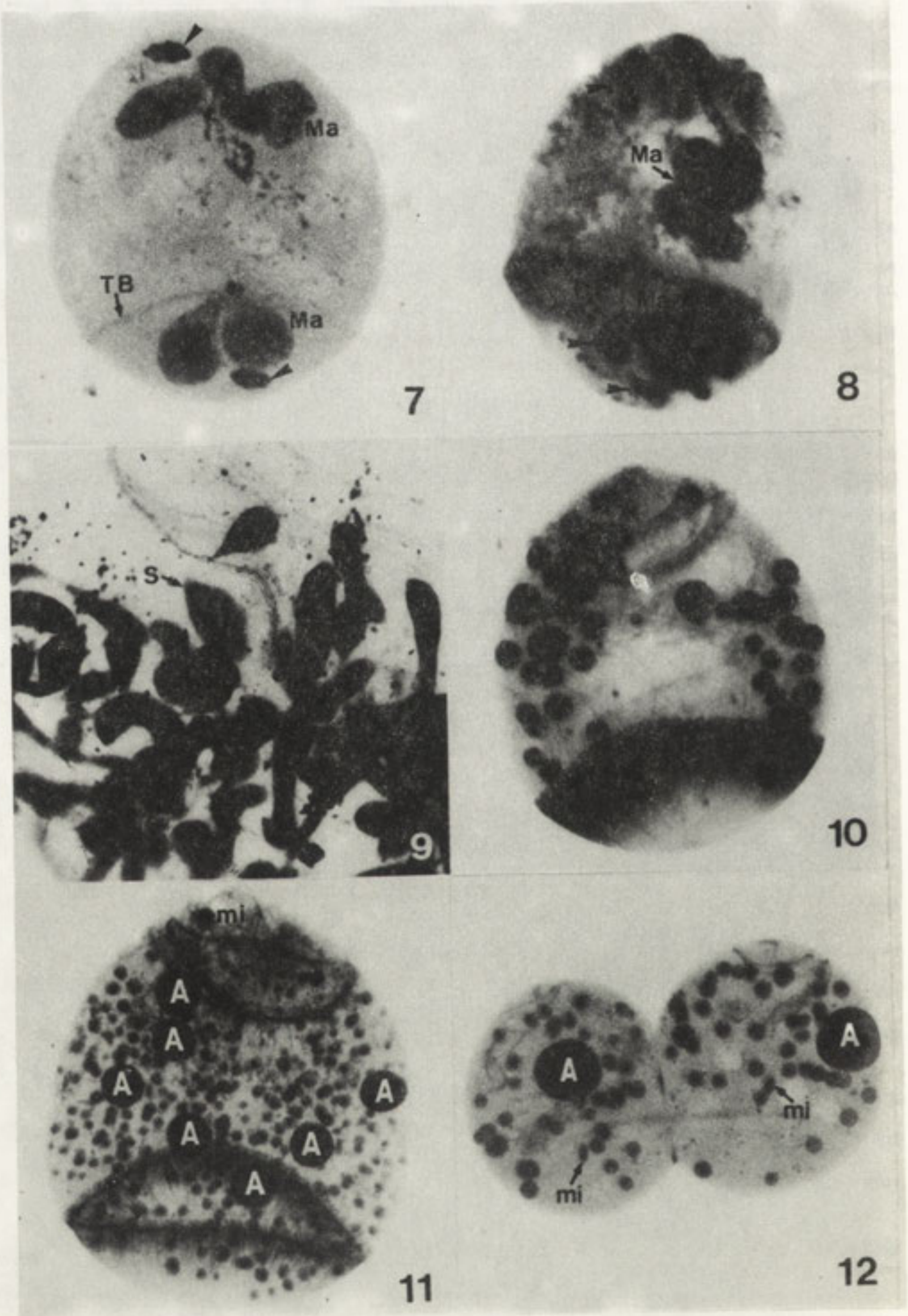
1. Preconjugation phase: formation of the macro-(Mc) and microconjugant (mc). Ma — macronucleus; mi — micronucleus; Sc — scopula; TB — trochal band; BI — buccal infraciliature. (× 550).
2. Penetration of the complete microconjugant cell (mc) into the macroconjugant (Mc). (× 475)
3. Detail of the trochal bands (TB) in both conjugants (Mc and mc). Ma — macronucleus. (× 1150)
4. The microconjugant cell (mc) is located in the aboral region of the macroconjugant cell (Mc). (× 725)
5. Micronuclear preliminary division in the microconjugant (point of arrow) (× 525)
6. Microconjugant with two micronuclei (points of arrow). (× 525)
7. First pregamic division of the micronuclei in both conjugants (points of arrow). Ma — macronucleus; TB — trochal band. (× 475)
8. Second pregamic division of the micronuclei of both conjugants (points of arrow) and macronuclear degeneration. Ma — macronucleus. (× 550)
9. Synkarion (S) located in the oral pole of the macroconjugant cell and macronuclear fragmentation. (× 1550)
10. Macronuclear degeneration and postzygotic synkarion divisions. (× 650).
11. Postconjugant cell with seven Anlagen (A) and one micronucleus (mi). (× 550)
12. Macronuclear segregation (1 : 1) during a postzygotic division of the cell. A — Anlagen. (× 325)

http://rcin.org.pl



A. Sola et al auctores phot.

http://rcin.org.pl



A. Sola et al.

auctores phot.

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 245-252 (1989)

Effects of Cadmium on Growth, Ultrastructure and Content of Chemical Elements in *Tetrahymena pyriformis* and *Acanthamoeba castellanii*

Wanda KRAWCZYŃSKA[1], Natasha N. PIVOVAROVA[2] and Andrzej SOBOTA[1]

[1] Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., 02-093 Warszawa, Poland; [2] Sechenov Institute of Evolutionary Physiology and Biochemistry, 46 Thorez Pr., 194223 Leningrad, USSR

Received on 17th October, 1988

Synopsis. The toxic effects of cadmium, the main bivalent cationic environmental polutant, on freshwater *Tetrahymena pyriformis* and soil *Acanthamoeba castellanii* were studied.

The growth of *Tetrahymena* culture was inhibited in the presence of 30 μM Cd^{2+}, whereas the growth of *Acanthamoeba* culture was much less sensitive to Cd^{2+} action and occured even at 100 μM Cd^{2+}. X-ray microanalysis demonstrated that cadmium was accumulated in *Tetrahymena* cells (2.4 mmoles/kg of wet weight) and in *Acanthamoeba* cells (0.7 mmoles/kg of wet weight). Simultaneously, in these organisms increased amounts of sulphur were detected. The electron microscope examination showed that cadmium caused several abnormalities in the ultrastructural organization of *Tetrahymena* cells, while no changes of ultrastructure of *Acanthamoeba* cells were visible.

The presented data indicate that *Acanthamoeba* cells are more resistant to the toxic effects of cadmium ions than the cells of *Tetrahymena*.

Cadmium is one of the most toxic bivalent heavy metals, whose concentration in environment tends to increase far above the natural level. In nature, this element accumulated greatly in sea organisms (Ray 1984). Less is known about the accumulation of cadmium in freshwater inhabitants. Freshwater protozoa are often used as a model system in studies of cadmium effects on physiological processes of cells and their ultrastructural organization (Ord and Al-Altia 1979; Iftode et al. 1985; Piccini et al. 1987). The studies on protozoa are important,

http://rcin.org.pl

since these organisms are involved in various freshwater food chains transferring the toxic elements to animals and human beings. Besides, the protozoa are very convenient material for studies of the effects of heavy metals at cellular level.

In the present paper we are dealing with the influence of cadmium on the population growth of *Tetrahymena* and *Acanthamoeba* culture, on the ultrastructural organization as well as the content of chemical elements.

Material and Methods

Two kinds of free-living unicellular organisms: a freshwater *Tetrahymena pyriformis* and a small soil amoeba *Acanthamoeba castellanii* were used to study the effects of cadmium on protozoa cells.

Tetrahymena pyriformis GL cells were grown axenically in the medium containing 1% proteose peptone and 0.1% yeast extract. A fresh 100 ml growth medium was innoculated with one drop of cells at a stationary growth phase. Usually after 2 days, when the culture attained the early logarithmic phase of growth, the cells were divided into equal portions and each of them was supplemented with cadmium chloride ($CdCl_2 \times 2.5\ H_2O$) at final concentration of 1, 10, 30 and 100μM. The growth of *Tetrahymena* populations was estimated by counting the cells in a Fuchs--Rosenthal haemocytometer.

Acanthamoeba castellanii (Neff strain) cells were cultured axenically in 100 ml Erlenmayer flasks containing 25 ml of optimal growth medium: 1.5% proteose peptone, 0.15% yeast extract, 1% glucose and salts as described by Sobota et al. (1984). The tested cadmium chloride solutions were added to the 2 days old *Acanthamoeba* culture at final concentration of 1, 10 and 100 μM. Cells were counted in a Thoma-Zeiss haemocytometer.

X-ray microanalysis of elements

The content of cadmium and other biologicaly important elements (Na, K, Ca, P, S) in the cells was analysed by quantitative x-ray microanalysis. The cells were harvested by mild centrifugation at 500 g for 3 min, rinsed either with 10 mM Tris-acetate buffer, pH 7.2 (*Tetrahymena*) or with 120 mB $LiNO_3$ (*Acanthamoeba*), then dried at 105°C until the cell samples reached a constant weight. The "cell dry powder" was placed in a round cavity in a carbon holder, coated with thin carbon layer, and the elemental analysis was carried out with x-ray microanalyser MS-46 (Cameca, France) under the following conditions: accelerating voltage 20 kV, sample current 50–60 nA, and the electron beam focused to a spot of 10 μm of diameter. A quantitative analysis was performed by comparing the signals from the experimantal material to the signals of cristal standards (Burovina and Pivovarova 1978).

Electron microscope procedure

The cells were fixed in 2.5% glutaraldehyde buffered with 100 mM cacodylate buffer, pH 7.2 for 1 h. Then the cells were rinsed in buffer and postfixed in 1%

http://rcin.org.pl

osmium tetroxide buffered as above. After washing and ethanol dehydration the cells were embedded in EPON 812. Ultrathin sections were cut with LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined with JEOL JEM-100 B electron microscope.

Results and Discussion

Cadmium ions added to the 2 days old *Tetrahymena* culture inhibited further growth of the culture in a dose-dependent manner (Fig. 1). When the cultures were exposed to 1μM cadmium ions, no significant changes in their growth were observed. About 30% of inhibition of the population growth was detected in the presence of 10 μM cadmium concentration. A very strong inhibition of the growth, nearly 100%, was found when the cells were treated with 30 μM cadmium chloride. The latter dose seemed to be lethal one under our experimental conditions.

The presented results agree with the observations of Pyne et al. (1983), who have shown that the concentration of 25 μM Cd^{2+} was sublethal one for *Tetrahymena* cells. However, Pyne's experiments were carried out in the defined Rosenbaum's medium (Rosenbaum et al.

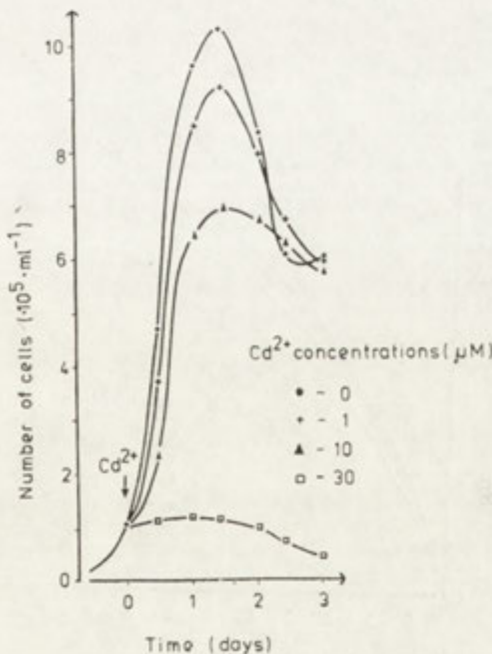


Fig. 1. Effect of cadmium on the growth of *Tetrahymena pyriformis* population. The cultures were supplemented with cadmium at the time indicated by arrow

http://rcin.org.pl

1966). When *Tetrahymena* cells were cultured on proteose peptone-yeast extract medium, the cells were much more sensitive to the cadmium toxicity (Pyne et al. 1983). The protective role of the defined Rosenbaum's medium against cadmium action also was noted by Fleury et al. (1983). In their experiments a dose of 10 μg/ml (it corresponds to ca 54 μM) was lethal one when *Tetrahymena* cells were maintained in the proteose peptone — yeast extract medium, whereas in the defined Rosenbaum's medium about 50% of the cells managed to survive in the same concentration of cadmium.

The effects of different concentrations of cadmium ions on the growth of *Acanthamoeba castellanii* culture is shown in Fig. 2. The low concentration of cadmium (1 μM) did not change the growth of the culture. The slight inhibition of the growth was observed in the presence of 10 μM Cd^{2+}, whereas the more pronounced reduction of the culture growth was found in the presence of 100 μM cadmium. In the cultures maintained for 7 days in the latter concentration of cadmium, about 30% of cells were in cyst forms.

An electron microscope examination of the ultrastructural organization of Cd-treated *Acanthamoeba* cells revealed no abnormalities in our experimental conditions. Ord and Al-Altia (1979) treating shortly

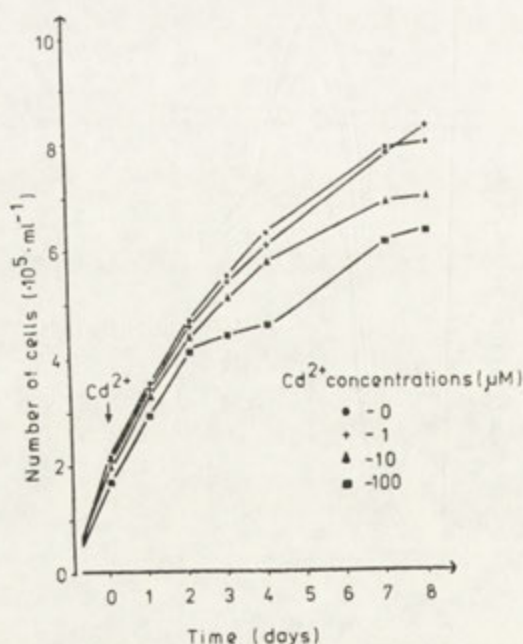


Fig. 2. Effect of cadmium on the growth of *Acanthamoeba castellanii* population. The cultures were supplemented with cadmium at the time indicated by arrow

http://rcin.org.pl

freshwater *Amoeba proteus* with a high concentration of cadmium, observed significant changes in the fine organization of mitochondria, ergastoplasmic reticulum and nuclei, as well.

An ultrastructural study of *Tetrahymena* cells exposed to 10 μM Cd^{2+} for 2 days revealed some changes in the cytoplasm and nucleus similarly to that described by Dunlop and Chapman (1981) and Pyne et al. (1983). In macronucleus, after 2 days of incubation of *Tetrahymena* cells with 10 μM Cd^{2+} some changes in the fine nucleolar organization are observed (Pl. I a, c) as compared with control (Pl. II a). Nucleoli showing irregular shape are composed mainly of fibrillar material, suggesting that an inhibition of ribosomal RNA synthesis took place. Such correlation was indicated by electron microscopical and biochemical studies of Iftode et al. (1985). In cytoplasm an increased number of irregularly shaped autophagic vacuoles filled with membranous material are seen (Pl. I a). Besides, increased amounts of lipid droplets as well as dense granules surrounded by trillamellar membrane are found (Pl. I b). In the cells exposed to 100 μM cadmium for 1 h the disintegration of mitochondrial membranes is detected (Pl. I d), as compared with untreated control (Pl. II b). However, it should be noted that ultrastructural changes observed in *Tetrahymena* cells exposed to 10 μM cadmium for 2 days were similar to those described in 7 days old, that is stationary phase cells (Levy and Elliot 1968).

The elemental analysis of *Tetrahymena* and *Acanthamoeba* cells exposed to cadmium ions demonstrated that an accumulation of that element inside the cells took place. High amount of cadmium — 2.4 mmoles per kg of wet cells was detected after 2 days of exposure of *Tetrahymena* cells to cadmium, whereas only about 0.7 mmoles of cadmium per kg of

Table 1

Content of chemical elements in *Tetrahymena pyriformis* and *Acanthamoeba castellanii* treated with cadmium

Cells	t	n	Cd	K	Na	Ca	P	S
			mmole/kg wet cell weight*					
Tetrahymena								
control	2 d	5	0	29.1±2.3	2.1±0.3	7.8±0.5	101.0±5.0	8.9±1.6
Cd^{2+}, 10 μM	2 d	5	2.4±0.2	33.3±1.5	1.5±0.3	7.1±0.4	104.1±8.0	10.8±1.5
Acanthamoeba								
control	1 d	3	0	40.0±1.3	20.5±2.5	2.1±0.1	95.3±6.7	2.9±0.3
Cd^{2+}, 1 μM	1 d	2	0	33.0±0.4	14.8±2.0	2.0±0.1	83.2±6.8	3.5±0.5
Cd^{2+}, 100 μM	1 d	3	< 0.7	31.8±2.5	15.6±1.6	1.9±0.2	73.4±7.6	3.6±0.5
Cd^{2+}, 100 μM	1.5 h	3	0	37.7±0.6	18.7±2.1	2.0±0.1	83.5±11.5	2.9±0.4

t — time of cell exposition to cadmium: d — day, h — hour; n — number of samples, each sample was measured in 10-12 spots; * — mean ± SD

http://rcin.org.pl

Kusamran K., Mattox S. M. and Thompson G. A. 1980: Studies on the size, location and turnover of calcium pools accessible to growing *Tetrahymena* cells. Biochem. Biophys. Acta, 598, 16–26.

Larochelle J. and Gagnon A. 1976: Osmoregulation in *Acathamoeba castellanni*. II. Variations of the concentrations of some intracellular ions. Comp. Biochem. Physiol., 54A, 275–279.

Levy M. R. and Elliot A. M. 1968: Biochemical and ultrastructural changes in *Tetrahymena pyriformis* during starvation. J. Protozool., 15, 208–222.

Martin B. 1984: Inorganic chemistry of calcium. In: Metal Ions in Biological Systems, (eds. H. Siegl and A. Siegl), 17, Marcel Dekker Inc., New York, 1–49.

Ord M. J. and Al-Altia G. R. 1979: The intracellular effect of cadmium: an experimental study using *Amoeba proteus* as a single cell model. In: The Chemistry, Biochemistry and Biology of Cadmium, (ed. M. Webb), Elsevier Biomedica Press, North Holland, 141–173.

Piccini E., Irato P., Soppellotti O. and Guidolini L. 1987: Biochemical and ultrastructural data on *Tetrahymena pyriformis* treated with copper and cadmium. J. Cell Sci., 88, 283–293.

Pyne C. K., Iftode F. and Curgy J. J. 1983: The effects of cadmium on the growth pattern and ultrastructure of the ciliate *Tettrahymena* and the antagonistic effect of calcium. Biol. Cell. 48, 121–131.

Ray S. 1984: Bioaccumulation of cadmium in marine organisms. Experientia, 40, 14–23.

Rosenbaum N., Ervin J., Beach D. and Holtz G. G., Jr. 1966: The induction of phospholipid requirement and morphological abnormalities in *Tetrahymena pyriformis* by growth at supraoptimal temperatures. J. Protozool., 13, 535–547.

Rugstad H. E. and Norseth T. 1975: Cadmium resistance content of cadmium-binding protein in cultured human cells. Nature, 257, 136–137.

Sobota A., Burovina I. V., Pogorelov G. and Solus A. A. 1984: Correlation between potassium and phosphorus content and their uniform distribution in *Acanthamoeba castellanii*. Histochemistry, 81, 201–204.

Webb M. 1979: The metallothioneins. In: The Chemistry, Biochemistry and Biology of Cadmium, (ed. M. Webb), Elsevier Biomedical Press, North Holland, 195–266.

Yamaguchi N., Wada O., Yazaki K. and Toyokawa K. 1973: Detection of heavy metals toxicity by *Tetrahymena pyriformis* culture method. Ind. Hlth, 11, 27–31.

EXPLANATION OF PLATES I–II

Ultrastructure of *Tetrahymena pyriformis* cells treated with cadmium; a–c — 10 μM Cd^{2+}, 2 days, d — 100 μM Cd^{2+}, 1 h

a: Fragment of the cell with macronucleus (Ma) containing irregular nucleoli (Nu). In cytoplasm lipid droplets (L), autophagic vacuole filled with membraneous material (AV) and dense bodies (D) are seen. × 19,100

b: Dense body surrounded by trilamellar membrane (arrow). × 43,700

c: Irregularly shaped nucleolus is filled with fibrillar material (f). At its periphery granular material (g) is seen. × 43,700

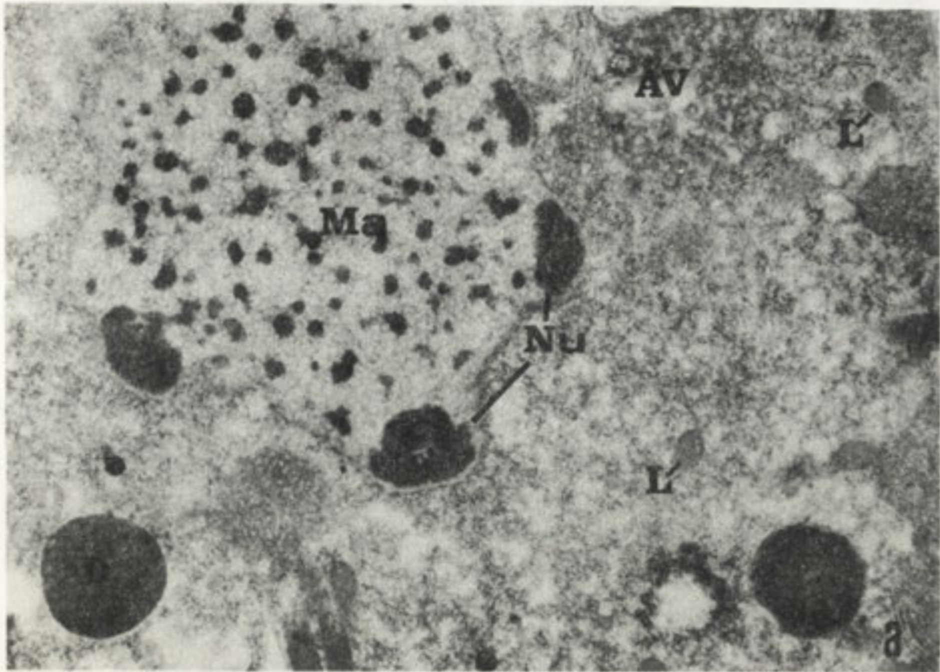
d: Mitochondrium: disintegration of crists and the outer mitochondrial membrane (arrow) is seen. × 38,600

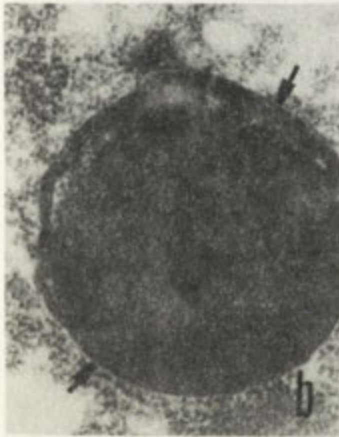
Ultrastructure of Cd-untreated *Tetrahymena pyriformis*.

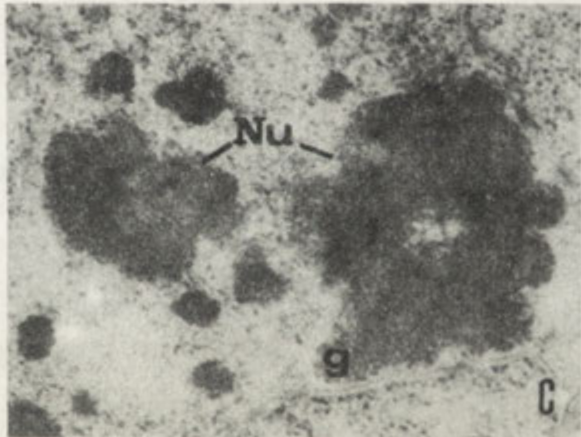
a: Fragment of the macronucleus (Ma) with bean-shaped, compact nucleoli (Nu). × 15,500

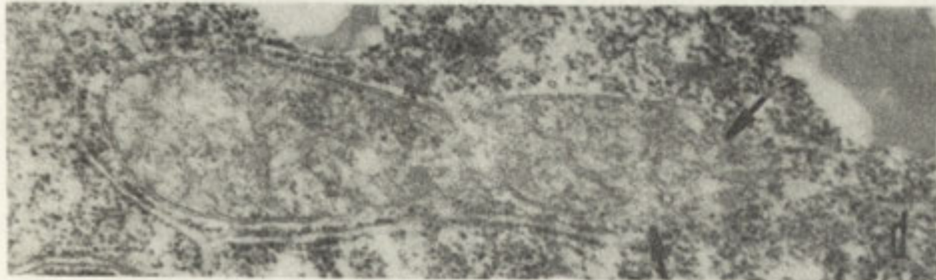
b: Mitochondrium with well organized crists. × 26,000

http://rcin.org.pl



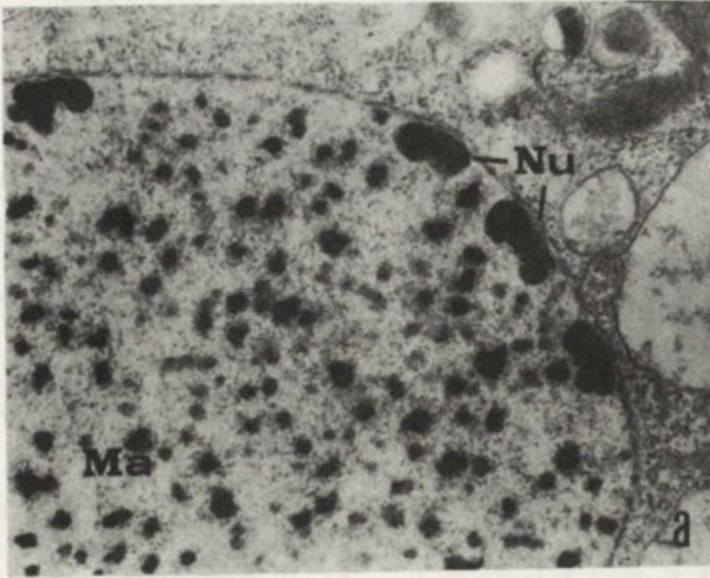






W. Krawczyńska et al. auctores phot.

http://rcin.org.pl



W. Krawczyńska et al. auctores phot.

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 253-263 (1989)

Modulators of Intracellular Sodium Concentration Affect Contractile Vacuole Activity in Ciliate *Blepharisma japonicum*

Hanna FABCZAK and Stanisław FABCZAK

Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., 02-093 Warszawa, Poland

Received on 17th October, 1988

Synopsis. The mode of action of externally applied sodium ions, Na^+-filled liposomes, or drugs (amiloride, monensin and ouabain) on the function of contractile vacuole in a fresh-water protozoa *Blepharisma japonicum*, was examined. When sodium ions or Na^+-filled liposomes were added to the cell medium, there was a rapid decrease in vacuolar pulsation rate with a progressive recovery on washing. A diuretic amiloride, a potent and specific inhibitor of sodium transport, enhanced the pulsatory rhythm of contractile vacuole by about 80% at concentration of 10^{-4} M. A Na^+-specific ionophore, monensin, at concentration of 10^{-5} M produced reversible decrease in frequency of pulsation of contractile vacuole, the effect being significantly dependent on the external Na^+ level. Ouabain, an evident Na^+/K^+ pump inhibitor, decreased the frequency of contractile vacuole pulsation as concentration of the drug increased, and ceased the vacuole functioning completely at concentration of 10^{-3} M. The results demonstrate that sodium ions and Na^+-containing liposomes as well as the used drugs have a profond effect on sodium homeostasis in ciliate cells, which is presumably maintained mainly by the contractile vacuole complex.

The contractile vacuoles are subcellular organelles with structure defined by their behavior: slow filling and periodical rapid expelling of the contents from the cell interior (Wichterman 1987, Kitching 1956, Ahmad and Couillard 1974). The contractile vacuoles in protozoan ciliates are of two kinds: those with canals emptying into them, as in *Paramecium*, and those formed by the coalescence of smaller vacuoles into a large primary vacuole that discharges the fluid, as in *Blepharisma* (Wrześniowski 1869, Moore 1934). In *Blepharisma*, the contractile vacuole complex is formed within granula-free and cilia-free posterior end but not necessarily in exactly the same spot during successive fluid evacuations (Moore 1934).

http://rcin.org.pl

The contractile vacuole in *Blepharisma* pulsates with rather slow frequency: from half or one contraction per 1 min to one contraction every 3 min, depending on the ambient temperature, osmolarity, and ion composition of the surrounding medium (Zanyin-Gaw 1936, Reuter 1963, Seravin 1958, Osanai 1961 a, b, Czarska 1964, Dunham and Stoner 1967, Boggs and Wade 1972, McNeil and Perkins 1972, Organ and Bovee 1972, Pothier et al. 1987). At standard conditions the primary vacuole appears to reach usually a constant size before each fluid expulsion.

The main function of the contractile vacuole system in protozoa is supposed to be the cell osmoregulation, and more specifically, the ion regulation within cytoplasm (Prosser 1974, Chapman-Andresen and Dick 1962. Dunham and Child 1961. Rifkin 1973, Connolly and Kerkut 1983). In particular, it has been observed that contractile vacuole pulsations are connected with the coupled extrusion of Na^+ and water, and the vacuole content is enriched in sodium and deplated in potassium ions in respect to the cytosol (Bruce and Marshall 1965, Raze and Schoffeniels 1965, Hamoton and Schwartz 1976). Inhibition of contractile vacuole activity by hypertonic shocks elevates reversibly the intracellular sodium ion (Na_i^+) level (Dunham and Stoner 1967, Kropp 1971). Changes of the cytosolic Na^+ concentration by some drugs lead to an inverse relationship between Na_i^+ level in cytoplasm and the contractile vacuole activity (Frixone and Perez-Olvera 1985). The latter observation indicates that the role of contractile vacuole complex in cytoplasmic ion regulation as well as the role of monovalent ions in vacuolar activity of a free-living unicellular organisms, are still insufficiently understood and need more extensive investigation. To gain further insight into the mechanism governing the contractile vacuole kinetics in protozoans, the effects of external modulators of intracellular Na^+ level on the time course of contractile behavior of vacuolar structures in ciliate *Blepharisma* have been examined. A preliminary report concerning the effect of two drugs, monensin and amiloride, known by their ability to alter membrane permeability to Na^+, on rhythm of contractile vacuole pulsation has appeared previously (Fabczak et al. 1988).

Material and Methods

Cells

Experiments were carried out on fresh-water ciliate cells, *Blepharisma japonicum*. The ciliates were cultivated in Pringsheim solution containing 0.1 mM

http://rcin.org.pl

Na_2HPO_4, 0.35 mM KCl, 0.08 mM $MgSO_4$, and 0.85 mM $Ca(NO_3)_2$ at pH 7.4 with addition of *Tetrahymena pyriformis* cells as food source under semidark conditions at room temperature. Cells chosen for each experiment were preincubated for 3 h in fresh Pringsheim medium without nutritional components.

Test Solutions

Monensin (Sigma) was at first dissolved in 96% ethanol to obtain 10 mM stock solution. Amiloride (Huthamaki. Finland) and ouabain (Serva) were separately dissolved in distilled water giving 10 mM stock solutions as well. Adequate amounts of stock solutions were added to the Pringsheim medium immediately before cell incubation.

The test solutions with high sodium, sucrose, or Na^+-filled liposomes were prepared by addition of proper amounts of NaCl, sucrose or a volume of desalted mixture of Na^+-filled liposomes to Pringsheim medium.

Liposome Preparation

Liposomes containing sodium ions were prepared according to Batzri and Korn (1973) method modified by Fabczak (1986). Briefly, an ethanol solution containing egg lecithin (Merck), lysolecithin (Sigma) and sterylamine (Sigma) in 10 : 0.5 : 1 molar ratio was rapidly injected with Hamilton syringe into Ca^{+2}-free Pringsheim medium, or into the same solution but supplied with 0.2 mM or 1M NaCl. The obtained liposome suspension was concentrated for 15 min on Amicon YM-100 membrane with rapid stirring under N_2 pressure of 0.2 MPa and then washed three times with Ca^{+2}-free Pringsheim solution on Amicon ultrafiltration device as well. The prepared liposome suspension was used for experiments immediately or was stored in ice for no longer than 1 h.

Measurements

Duration of pulsation cycles of contractile vacuole under different tested conditions was determined with an Ergaval light microscope (Zeiss) at low magnification in ciliate cells placed under a cover-glass sealed to the slide with vaseline. For each testing solution few cells were selected at random from the Pringsheim medium, and time required for one vacuolar cycle or pulsation rate per min was measured with stop watch. The data of measurements on 10 to 15 cells were averaged for each experimental point presented in the graphs.

Results

Sodium Ions and Na^+-Filled Liposomes

In *Blepharisma* cells incubated in the Pringsheim solution at room temperature, the vacuoles contracted at an average rate of one cycle in two minutes (Fig. 1. at 0.2 mM Na^+). The addition of NaCl to the control solution at final concentration of 5 to 15 mM caused a progressive decline in frequency of the vacuolar content extrusion. At external sodium concentration (Na_0^+) of 15 mM the inhibition amounts up to 80% of initial rate (Fig. 1b). The higher concentrations of Na_0^+ were not used since

http://rcin.org.pl

incubation and subsequent cell washing did not lead to complete recovery of the vacuole contraction activity. The non-electrolytic solutions were less effective than electrolytes in suppressing the contractile vacuole activity. A complete suppression of vacuolar pulsation could be achieved at sucrose concentration of about 0.1 M in the cell medium.

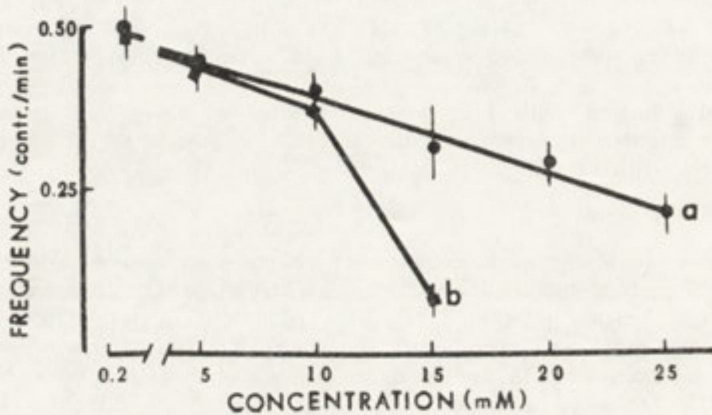


Fig. 1. Inhibition of contractile vacuole function following treatment of *Blepharisma* with different sodium (b) or sucrose (a) concentrations

The liposomes containing Na^{+} in concentration of 0.2 mM and 1.0 M in the tested solution were used to study their effect on vacuolar activity in *Blepharisma* cells as well. Figure 2b demonstrates that within several minutes after addition Na^{+}-filled liposomes (5% v/v) to the Pringsheim solution the frequency of vacuole contractions decreased substantially as compared with the control level. After a 10 min incubation,

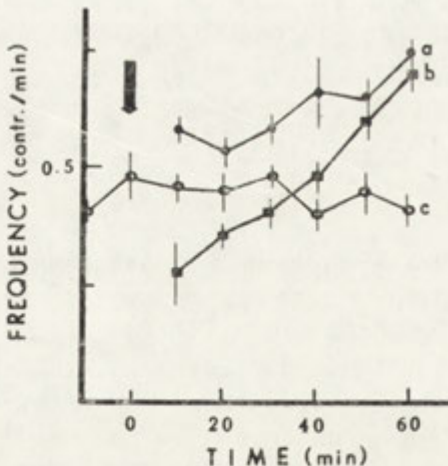


Fig. 2. The effect of "empty" (0.2 mM NaCl, a) or sodium filled (1.0 M NaCl, b) liposomes added to Pringsheim solution (c) on the activity of the contractile vacuole in *Blepharisma*. Arrows in this and the following figures indicate the onset of exposure to the test solutions

http://rcin.org.pl

a progressive enhancement of contraction rhythm occurred up to much higher level than the control one (in Pringsheim solution). The "empty" liposomes (Fig. 2a), which contained sodium ions at concentration of Pringsheim solution (0.2 mM), did not suppress the contractile activity of vacuoles, but on the contrary the permanent rise in activity occurred during incubation attaining in 60 s much higher value than the control one (i.e., 0.75 contraction per min). The control rhythm of vacuolar activity was restored in 10 to 15 min after washing.

Amiloride

The contractile vacuole activity increased twice in medium with amiloride in comparison with the control value (Fig. 3a). The reaction to amiloride was almost immediate but not very pronounced. An increase in Na_0^+ concentration of amiloride-containing solution caused an immediate decrease in frequency of vacuole contractions, however depending on the external Na_0^+ level (Fig. 4 c, d). Washing of cells from both sodium ions and amiloride, and subsequent incubation in fresh Pringsheim solution restored the normal vacuolar contractile activity. Amiloride in concentration lower than 10^{-4} M did not cause any noticeable changes in activity of cell vacuolar system. None of the tested amiloride concentrations did change the cell motility.

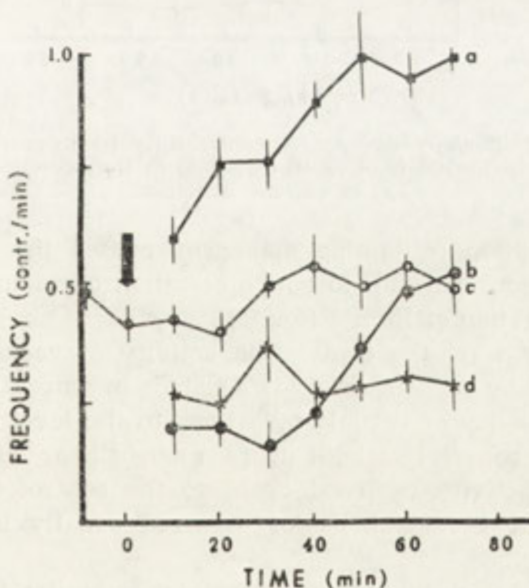


Fig. 3. Modulations of frequency contraction of vacuole in *Blepharisma* by sodium ionophore monensin (b), amiloride (a), or ouabain (d) added to Pringsheim solution (c)

http://rcin.org.pl

Monensin and Ouabain

Figure 3b shows in turn the reaction of cells to monensin supplied to the incubation solution. Monensin at concentration of 10^{-5} M caused a significant and rapid decrease in contractions frequency up to almost 0.2 contraction per minute (about 50% of the control) with following spontaneous recovery to the control level within 40 min. Sodium ions

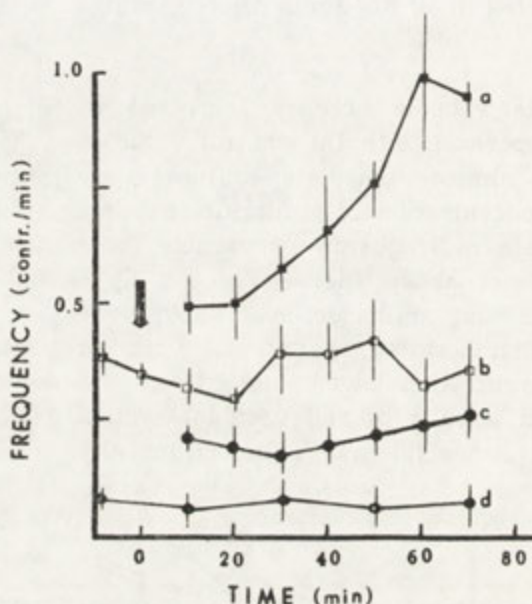


Fig. 4. Inhibition of *Blepharisma* vacuole contractility by increase of external sodium concentration to 10 mM (a,b) or 15 mM (c,d) in the presence (b,d) or absence (a,c) of diuretic amiloride

added to the solution containing monensin, caused the further rise of vacuolar contraction cycle up to entire cessation of vacuole pulsation at the Na_0^+ concentration of 15 mM (Fig. 5).

In the similar way, the contraction activity of vacuoles in *Blepharisma* is modified by ouabain (Fig. 3d). Ouabain in concentration of 10^{-3} M reduced the frequency of vacuole pulsations to the level of 60% of control value. After removal of both drugs, monensin or ouabain, the contractile vacuole activity returned again to the control level. Complete renormalization of vacuole behavior occurred usually in 5 to 10 min after cell washing.

Cells incubated with monensin, in contrast to amiloride, were characterized by very slow swimming, and an apparent slight increase in vacuole diameter at full diastole was evident in some organisms.

http://rcin.org.pl

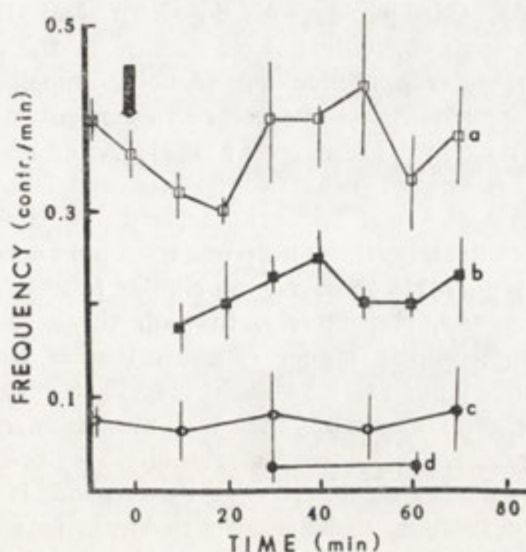


Fig. 5. The effect of extracellular sodium (10 mM a,b or 15 mM c,d) on contractile vacuole activity in *Blepharisma* in the absence (a,c) and presence (b, d) of ionophore monensin

Discussion

Vacuole in *Blepharisma* contracts at rather long intervals, on an average half vacuolar cycle per minute (Fig. 1a, Zanyin-Gaw 1936). For comparison, the frequency of pulsation of contractile vacuoles in *Paramecium* is about 7 to 9 cycles per minute (Boggs and Wade 1972, Frixione and Perez-Olvera 1985). Frequency of vacuole pulsations can be changed by the external factors such as temperature (Cole 1925, Ahmad and Couillard 1974), ion concentration or ion composition of the medium (Osanai 1961 a, b, Reuter 1963, Czarska 1964, Organ and Bovee 1972, Rifkin 1973). Contractile vacuole activity depends not only on the osmolarity of the medium since far more strong effect of sodium on vacuole pulsation is apparent in comparison with that of equivalent molar concentration of sucrose (Fig. 1a). That specific effect of sodium can be caused by increase in intracellular Na^+ level as a result of increase of sodium ion concentration in the medium (Hilden 1970).

The close relationship between the frequency of vacuole concentration in protozoan cells and the level of cytoplasmic Na_i^+ was suggested by several authors involved in studying of that problem (Dunham

http://rcin.org.pl

and Child 1961, Chapman-Andresen and Dick 1962). In cells treated with high concentration of sucrose in the medium (hypertonic shock), complete suppression of vacuolar expelling activity was observed as well as simultaneous increase in concentration of cytoplasmic sodium (Dunham and Stoner 1967). After removal of sucrose from the medium, the normal rate of contractile vacuole activity in *Tetrahymena* was restored and intracellular sodium concentration also returned to the control level. Chapman-Andresen and Dick (1962) and Kropp (1971) observed the similar process in *Tetrahymena* and *Chaos carolinensis*, respectively. In both those cells the vacuolar fluid contained significantly higher concentration of Na^+ than that of the cytoplasm (Schmidt-Nielsen and Schrauger 1963, Riddick 1968). The electrophysiological measurements showed that the vacuolar content was positive in relation to cytoplasma in studied protozoan cells (Yamaguchi 1960, Prush and Dunham 1970). Thus, the above data allow to believe that sodium ions are actively expelled against the electrical and chemical gradients and that the vacuolar contractile mechanism thereby takes part in regulation of cytoplasmic Na^+ level in protozoa.

However, the recent data of Frixione and Perez-Olvera (1985) in *Paramecium* as well as the results of experiments on *Blepharisma* presented in this paper indicate the close relations between vacuole contractility and internal sodium though the nature of the relations seems to be more complicated. The external factors which lead to the elevation of cytoplasmic Na^+ level (i.e., high Na_0^+, ouabain, monensin, liposomes containing high Na^+-ion concentrations) induce a temporary or permanent decrease in rate of vacuolar activity in ciliates (Fig. 1, 2, 3). On the other hand, the factors causing a decrease in cytoplasmic Na^+ level (i.e., amiloride,) enhance unquestionably the vacuole pulsation (Fig. 3, 4). Thus, there is a relation between Na_i^+ level and the time course of vacuolar activity, while a conclusion on straight regulation of contractile vacuole activity by Na_i^+ cannot be drawn. The interpretation of Frixione and Perez-Olvera (1985) seems to be reliable, that sodium dependent changes of concentration of free cytoplasmic calcium, which is involved in regulation of the most motile phenomena in non-muscle cells (Eckert et al. 1976, Hitchcock 1977), are responsible for the observed relationship between Na_i^+ and activity of vacuole contractile system. In protozoa that type of ionic regulation has not been found yet, although its existence could be assumed since antiport Na^+/Ca^{2+} exchange mechanisms, at plasma membrane are known in several kinds of excitable cells, which are responsible directly for cytoplasmic Ca^{2+} regulation (Glitsch et al. 1970, Blaustein 1974,

http://rcin.org.pl

Chapman et al. 1983, DiPolo and Beauge 1983, Liu et al. 1985, Jacob et al. 1987).

It is also found that the rate of contractile vacuole activity in *Blepharisma* is significantly changed by addition of the ouabain, modulator of Na^+/K^+ pump, (Fig. 3d) in the similar way as in *Paramecium* (Poggs and Wade 1972). This and potassium/sodium ions relation at both sides of vacuolar membrane (i.e., in vacuolar and cytoplasmic fluids) (Schmidt-Nielsen and Schrauger 1963, Bruce and Marshall 1965, Hilden 1970), suggest an existence of Na^+/K^+ exchange at vacuolar membrane. Localization of Na^+/K^+-ATPase in vacuolar membranes associated with the major Na^+ extrusion from the protozoan cells is postulated elsewhere (Connolly and Kerkut 1983).

In conclusion, the experimental results presented in this paper indicate that intracellular Na^+ concentration changes bring about the considerable effect on contractile activity of vacuole. Elevation of cytosolic sodium decrease the frequency of vacuole contraction in *Blepharisma*, whereas lowering has an opposite effect, what is in evident disagreement with the present hypothesis that coupling between sodium and water extrusion might be directly involved in the regulation of contraction of vacuolar complex (Dunham and Child 1961, Raze and Schoffeniels 1965, Hampton and Schwartz 1976, Pothier et al. 1987). The complete elucidation of the observed relations as well as the precise definition of possible role of ion exchange of Na^+/Ca^{2+} or Na^+/K^+ type in regulation of vacuole contractility is still impossible at present stage of knowledge.

ACKNOWLEDGEMENTS

We would like to thank Dr. Jerzy Sikora for his critical reading of the manuscript.

REFERENCES

Ahmad M. and Couillard P. 1974: The contractile vacuole in *Amoeba proteus*: Temperature effects. J. Protozool. 21, 330–336.

Batzri S. and Korn E. D. 1973: Single bilayer liposomes prepared without sonication. Biochem. Biophys. Acta. 298. 1015–1019.

Blaustein M. P. 1974: The interrelationship between sodium and calcium fluxes across cell membranes. Rev. Physiol. Biochem. Pharm., 70 33–82.

Boggs N. Jr., and Wade W. E. 1972: The effect of ouabain on the water expulsion vesicle in *Paramecium multimicronucleatum*. J. Protozool. 19. Suppl., 34–35.

http://rcin.org.pl

Bruce D. L. and Marshall J. M. 1965: Some ionic and bioelectric properties of the amoeba Chaos chaos. J. Gen. Physiol., 49, 151–178.
Chapman-Andresen C. and Dick D. A. T. 1962: Sodium and bromide fluxes in the amoeba *Chaos chaos* L. C. R. Trav. Lab. Carlsberg, 32, 445–469.
Chapman R. A., Coray A. and McGuigan J. A. S. 1983: Sodium-calcium exchange in mammalian ventricular muscle: a study with sodium-sensitive microelectrodes. J. Physiol. (London), 343, 253–276.
Cole W. H. 1925: Pulsation of the contractile vacuole of *Paramecium* as affected by temperature. J. Bact., 84, 754–758.
Connolly J. G. and Kerkut G. A. 1983: Ion regulation and membrane potential in *Tetrahymena pyriformis*. Comp. Biochem. Physiol. 76A, 1–16.
Czarska L. 1964: Role of the K and Ca ions in the excitability of protozoan cell. Chemical and electric stimulation of contractile vacuoles. Acta Protozool. 2, 287–296.
DiPolo R. and Beauge L. 1983: The calcium pump and sodium-calcium exchange in squid axons. Annu. Rev. Physiol., 45, 313–324.
Dunham P. B. and Child F. M. 1961: Ion regulation in *Tetrahymena pyriformis*. Biol. Bull., 121, 129–140.
Dunham P. B. and Stoner L. C. 1967: Regulation of intracellular sodium concentration by the contractile vacuole in *Tetrahymena*. J. Protozool. 14, Suppl., 345.
Eckert R., Naitoh Y. and Machemer H. 1976: Calcium in the bioelectric and motor functions of *Paramecium*. Symp. Soc. Exp. Biol., 30. 233–256.
Fabczak H. 1986: Cholesterol-enriched liposomes affect food-vacuole formation in *Tetrahymena pyriformis*. Acta Protozool., 25, 325–331.
Fabczak H., Fabczak S. and Wadzyńska M. 1988: Wpływ wewnątrzkomórkowego sodu na aktywność kurczliwą wodniczki tętniącej u *Blepharisma japonicum*. III Ogólnopolska Konferencja Biologii Komórki. Łódź, 88.
Frixone E. and Perez-Olvera O. 1985: Contractile vacuole activity in *Paramecium aurelia*: dependence upon intracellular sodium. Comp. Biochem. Physiol., 82A, 787–790.
Glitsch H. G., Reuter H. and Scholz H. 1970: The effect of internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. J. Physiol. (London), 209, 25–43.
Hampton J. R. and Schartz J. R. L. 1976: Contractile vacuole function in *Pseudocohnilembus persalinus*: responses to variations in ion and total solute concentration. Comp. Biochem. and Physiol., 55, 1–4.
Hilden S. A. 1970: Sodium and potassium levels in *Blepharisma intermedium*. Exp. Cell. Res., 61, 241–254.
Hitchcock S. A. 1977: Regulation of motility in non-muscle cells. J. Cell Biol., 74, 1–15.
Jacob R., Lieberman M. and Liu S. 1987: Electrogenic sodium/calcium exchange in cultured embryonic chick heart cells. J. Physiol. (London), 387, 567–588.
Kitching J. A. 1956: Contractile vacuoles of protozoa. Protoplasmatologia, IIID3a, 1–45.
Kropp D. L. 1971: Mechanism of sodium infux in *Tetrahymena pyriformis*. Ph. D. Thesis. University of Syraciuse USA.
Liu S., Jacob R. Lieberman M. 1985: Electrogenic Na^{+}/Ca^{++} exchange in cultured chick heart cells demonstrated using ion-selective microelectrodes. Fed. Proc., 44, 1580–1586.
McNeil N. K. and Perkins D. L. 1972: Effect of constant Jahn ratios on the water expulsion vesicle activity of *Negleria gruberi*. J. Protozool. 19, Suppl., 40.
Moore I. 1934: Morphology of the contractile vacuole and cloacal region of *Blepharisma undulans*. J. Exp. Zool. 69, 59–104.
Organ A. E. and Bovee E. C. 1972: Water flux in *Tetrahymena pyriformis*. Are osmotic differentials important? J. Protozool. 19, Suppl., 27.
Osanai N. 1961 a: Effects of Na^{+}, K^{+}, Mg^{++} and Ca^{++} ions on the contractile vacuole of a fresh-water protozoa. Cothurnia rp. Zool. Mag., 70, 367–372.
Osanai N. 1961 b: Effect of osmotic pressure on the contractile vacuole of the fresh-water protozoa. Cothurnia rp. Zool. Mag., 70. 160–164.

http://rcin.org.pl

Pothier F., Forget J., Sulivan R. and Couillard P. 1987: ATP and contractile vacuole in *Amoeba proteus*: Mechanism of action of exogenous ATP and related nucleotides. J. Exp. Zool., 243, 379–397.
Prosser C. L. 1974: Smooth muscle. Annu. Rev. Physiol. 36, 503–535.
Prush R. D. and Dunham P. B. 1970: Contraction of isolated contractile vacuoles from *Amoeba proteus*. J. Cell Biol., 46, 431–434.
Raze C. and Schoffeniels E. 1965: Role de la vacuole contractile chez *Paramecium caudatum* Ehrenberg. Bull. Acad. R. Belg. Cl. Sci., 1057–1073.
Reuter J. 1963: The internal concentration in some hypotrichous ciliates and its dependence on the external concentration. Acta Zool. Fennica, 104, 1–94.
Riddick D. H. 1968: Contractile vacuole in the amoeba *Pelomyxa carolinensis*. Am. J. Physiol., 215, 736–740.
Rifkin J. L. 1973: The role of the contractile vacuole in the osmoregulation of *Tetrahymena pyriformis*. J. Protozool., 20, 108–114.
Schmidt-Nielsen B. and Schrauger C. R. 1963: *Amoeba proteus*: studying the contractile vacuole by micropuncture. Science, 139, 606–607.
Seravin L. N. 1958: Relationship of the activity of the contractile vacuole of *Paramecium caudatum* and environmental conditions. Vestnik Leningrad. Univ., 3, 77–95.
Wichterman R. 1987: The Biology of *Paramecium*. Plenum Press, New York and London.
Wrześniowski A. 1869: Ein Beitrage zur Anatomie der Infusorien. Arch. Mikr. Anat., 5, 25–48.
Yamaguchi T. 1960: Studies on the modes of ionic behaviour across the ectoplasmic membrane of *Paramecium*. I. Electric potential differences measured by intracellular microelectrode. J. Fac. Sci. Tokyo Univ., Sec. 4, 8, 575–591.
Zanyin-Gaw H. 1936. Physiology of the contractile vacuole in ciliates. Arch. Protistenk. 87, 185–193.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 265-271 (1989)

Application of Ion-Selective Microelectrode in Measurement of Intracellular Chloride Activity in Ciliate *Blepharisma japonicum*

Stanisław FABCZAK and Hanna FABCZAK

Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., 02-093 Warszawa, Poland

Received on 24th November, 1988

Synopsis. Steady-state membrane potential, E_m, and intracellular chloride activity, a_{cl}, were measured with single-barrelled Cl^--selective glass microelectrodes based on two different types of chloride liquid--exchangers, Corning No. 477315 and W.-P. Instruments No. IE-170, in protozoan *Blepharisma japonicum*. In Pringsheim bathing solutions the mean a_{cl} was 3.6±09 mM and the intracellular E_m was -40.8±1.8 mV (20.5°C). These indicated that the intracellular a_{Cl} of the cell was higher than that predicted by the Nernst equation for passive distribution of these ions (i.e., a_{Cl} = 2.03 mM) by about 1.6 mM what suggest the presence of the process responsible for accumulating Cl^- ions against their electrochemical gradient.

Development of chloride glass microelectrodes has allowed the continuous measurements of intracellular chloride activity in variety of cells in vivo (Kunze and Brown 1971, Russel and Brown 1972, Ziskind et al. 1975, Russel 1978, Moreton and Gardner 1981). The chloride level within the cell is of considerable interest to those attempting to resolve a problem of whether intracellular ionic chloride octivity, a_{Cl}, in excitable cells is actively regulated or controlled by passive ion distribution across the plasma membrane. For muscle cells it has been demonstrated that the intracellular chloride is far higher than that predicted from a passive distribution, when it would be governed by the extracellular chloride and membrane potential (Kernan et al. 1974, Bolton and Vaughan-Jones 1976, Vaughan-Jones 1977, Caille et al. 1981, Aickin and Brading 1982).

The first attempt to measure the intracellular chloride in protozoan cell, was made by Akita (1941) in *Paramecium* where chloride was

http://rcin.org.pl

not estimated in detectable level, even when the organisms were immersed for a considerable time in a medium rich in Cl^- ions. Subsequently it has been demonstrated that the intracellular levels of Cl^- ions of *Amoeba proteus* (9.7 mM) or *Stentor coeruleus* (9.4 mM) are higher than chloride concentration in medium or lower as for *Tetrahymena pyriformis* cells (6.4 mM) (Dunham and Child 1961, Prush and Dunham 1972, Wood 1982). On the basis of the foregoing data and in consideration of the indirect methods used for measuring of the internal chloride there is difficult to solve the question of chloride regulation in protozoan cells. Therefore, we set out to measure intracellular a_{Cl} directly in *Blepharisma* cells to obtain the preliminary data for studying the regulatory mechanism for those ions and to investigate the two types of the commercially available chloride liquid ion-exchangers (i.e., Corning No. 477315 and W.-P. Instruments No. IE-170) for intracellular ion activity measurements.

Material and Methods

Stock cell cultures of *Blepharisma japonicum* were maintained in Pringsheim medium at pH 7.2 under semi-dark conditions at room temperature. The food source for those cells were the cells of *Tetrahymena pyriformis* from the axenic cultures. The cells chosen for certain experiments were transferred to a fresh maintenance medium without nutritional components for a few hours and after subsequent medium exchange the cells were finally placed into an experimental teflon chamber.

Single-barrelled Cl^--selective glass microelectrodes were fabricated from 1.5-mm-outside diameter pyrex tubes (1B150F-4, W.-P. Instruments, USA) pulled with a horizontal electric puller to a resistance of 15–30 Mohms when filled with 2.0 M KCl solution. The obtained micropipettes were then siliconized by dipping the tips in a 2% solution (v/v) of dimethylchlorosilane in carbon tetrachloride and sucked up to a height of 450–500 μm from the tip. Afterwards the micropipettes were heated with the tips up in a predried oven at 150 to 200°C for 1 h. The siliconized micropipettes were filled, first by dipping the tips in Cl^--sensitive liquid exchanger (W.-P. Instruments, No. IE-170 or Corning No. 477315, USA), and then by backfilling the remaining tapered portion with more exchanger. The solution of 150 mM KCl was used as a reference medium. An electrical resistance the Cl^--selective microelectrode prepared in this way was approximately 10^9 to 10^{10} ohms. Each selective microelectrode was calibrated before and after experiment by recording the voltage changes in pure KCl solutions at following concentrations (activities): 100(70), 10(9), 1.0(1.0) and 0.1(0.1) mM (Dean 1985).

Membrane potential, E_m, was measured with a conventional glass microelectrode (30–50 Mohms) filled with the solution of 0.8 M K_2SO_4 and 0.2 M KCl. Intrarellular impalements were only accepted if either (a) there was an immediate jump in electrical potential measured by both conventional and ion-selective electrodes as they crossed the cell membrane, and (b) the potential recorded by each micro-

http://rcin.org.pl

electrode impaled into the cell for at least 10 min did not alter by more than 1 or 2 mV per min. After each microelectrode impalement, there was often a gradual hyperpolarizing creep of E_m presumably due to sealing around the site of impalement. For measuring ion activity, a_{Cl}, two methods were used. In the first the cells were impaled simultaneously with two microelecrodes, Cl^--selective and conventional one. Current passed through the conventional microelectrode produced a change in voltage recorded by Cl^--selective microelectrode, verifying that both electrodes were within cytoplasm of the cell. The potential difference between conventonal and Cl^--selective microelectrodes was then used for calculation of a_{Cl} from the electrode calibration curves. In the second method, intracellular potentials were sampled with each electrode in turn. First, 5 to 10 cells were punctured with E_m-recording electrode, and then other 5 to 10 cells from the same sample were impaled with Cl^--selective microelectrode. The potential difference between the electrical values of the sampled cells was used again for a_{cl} calculations.

The resistance of E_m-recording microelectrode was checked usually before and after the cell penetrations, and the measured membrane potential data were taken into account when no change in resistance was found. The liquid junction potentials for those microelectrodes were usually less than 5 mV. Ion-selective and conventional microelectrodes were connected to the electronic recording system by nonpolarizable Ag-$AgCl_2$ wires. The bath solution was coupled with the circuit via $AgCl_2$-KCl-filled agar bridge. The recorded fast and slow electrical responses delivered by the microelectrodes were displayed on an oscilloscope (5103N, Tetronix Inc., USA) or on a digital multimeter (V-650, Meratronik, Poland), respectively.

Results and Discussion

Calibration curves of the representative microelectrodes with two different types of liquid Cl-selective exchanger, Corning No. 477317 and W.-P. Instruments No. IE-170, in various calibrating solutions are shown in Fig. 1. In KCl solutions microelectrodes gave a virtually linear responses between 1 and 100 mM for Corning No. 477315 resin with the slope of 52.7 and for W.-P. Instruments, No. IE-170 of 56.8 mV per tenfold change in chloride concentration of calibration solutions at room temperature. However, significant deviations from linearity occurred in calibration solution of pure KCl below 0.1 mM KCl. The effect was more evident for ion-exchanger Corning No. 477315, when deviation from linearity occurred already below 10 mM KCl in calibration solutions of KCl with 10 mM $NaHCO_3$. Limit detection of chloride ions for both types exchangers was in a range of 0.1 mM KCl, if there were no interfering ions in calibration solutions.

When the solutions contained except KCl salt also 10 mM $NaHCO_3$, the microelectrode sensitivity lowered significantly, and the threshold of measuring was close to 1 mM KCl level. Sometimes the microelectrodes with chloride exchanger Corning No. 477315 demonstrated substan-

http://rcin.org.pl

tial low sensitivity for Cl^- in calibration solutions containing KCl only as well as in those containing KCl-$NaHCO_3$. The same phenomenon was never observed for microelectrodes with chloride liquid ion-exchanger No. IE-170 from W.-P. Instruments. The cause of such behaviour of ion exchanger Corning No. 477315 is not known, though it was often observed that HCO_3-anions made worse the measuring properties of Cl-selective microelectrodes of different types (Meier et al. (1982). Because of the better measuring characteristics of the microelectrodes containing

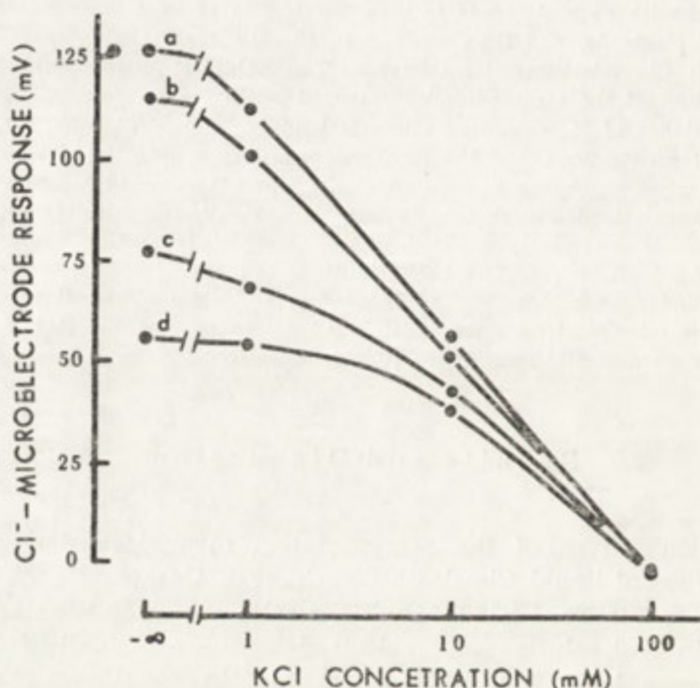


Fig. 1. Semilogarithmic plot of the electrical responses of Cl—-selective microelecteodes based on No. IE-170 (a, c) and No. 477315 (b, d) ion exchangers recorded in different concentration of pure KCl solutions (a, b) or in KCl with addition of 10 mM $NaHCO_3$ (c. d)

ion-exchanger of W.-P. Instruments No. IE-170, the last one were used for measuring the Cl^- level in cytoplasm of ciliate cells *Blepharisma japonicum*. Especially with *Blepharisma* cells, the sensitivity of microelectrodes with Corning exchanger was found to be insufficient, though the above fact was not so important for measurements of the internal Cl^- in the cells of multicellular organisms where the intracellular chloride activities were proved to be much higher there in *Protozoa* (Keynes 1963, Kerkut and Meech 1966, Kunze and Brown 1971, Caille et al. 1981).

http://rcin.org.pl

The values of intracellular activity, a_{Cl} and membrane potential, E_m, are presented in Table I as the means of 27 microelectrodes impalements of 14 *Blepharisma* cells obtained mainly by the second method described in Material and Methods. In Table I the a_{Cl} values on both sides of plasma membrane and a^{eq}_{Cl} value, resulting from the measured membrane potential, E_m, and extracellular concentration of Cl^- ions, assuming that

Table 1

Membrane potential, E_m, cytoplasmic, a_{cl}, and medium chloride, a_{cl}, activities and chloride level activity, a_{cl}, required for passive distribution of this ion a^{eq}_{cl}, in *Blepharisma*

E_m (mV)	a^i_{cl} (mM)	a^0_{cl} (mM)	a^{eq}_{cl} (mM)
-40.8 ± 1.8	3.6 ± 0.9	0.4	2.03

electrochemical activity of Cl^- ions were the same in all the experiments are given as well (Baumgarten 1981, Dean 1985). Thus, it is evident that *Blepharisma* cells in resting state contain Cl^- ions at the activity of 3.6 mM. that is about 1.6 mM higher than $a_{Cl}{}^{eq}$ predicted for a passive ion flux (i.e., 2.03 mM). The same phenomenon is observed in many cells of higher organisms. Much higher levels of internal chloride than it could be assumed from passive distribution of that ions across the cell membrane, were stated first of all in muscle cells (e.g., frog skeletal and sartorius muscles, heart muscle, rabbit papillary muscle, etc.) (Kernan et al. 1974, Bolton and Vaughan-Jones 1976, Vaughan-Jones 1979, Caille et al. 1981). The problem of mechanisms regulating the cytoplasmic Cl^- level in protozoan cells has not been clarified up to the present. It is only known that in the cells of a ciliate *Tetrahymena pyriformis* the intracellular concentration of Cl^- is far lower than Cl^- level in cell medium and than the level of cytoplasmic Na^+ (Dunham and Child 1961, Dunham 1964). Moreover, it has been shown for those organisms that cytoplasmic Cl^- did not change in a wide range of extracellular Cl^- concentrations (from 14 to 100 mM), though a simultaneous considerable rise of intracellular Na^+ or K^+ concentrations was observed when chloride level had been changed in the cell medium by means of NaCl or KCl salt. The authors have concluded that Cl^- was passively transported across the membrane, though expelling of that ions could take place by means of contractile vacuole (Dunham and Stoner 1967). The electrophysiological studies of a ciliate *Stentor coeruleus* revealed that changes in extracellular Cl^- level resulting from their exchange for NO_3^- or propionate, caused

http://rcin.org.pl

no essential change in membrane potential (Wood 1982), what could testify for the low permeability of *Stentor* membrane for Cl^- ions. It was also found that plasmalemma of amoeba *Chaos chaos* was not permeable for chloride, but that it might be supplied with the food (Bruce and Marshall 1965). However, similar studies on *Amoeba proteus* cells showed that cytoplasmic chloride was intensively exchanged with $^{36}Cl^-$ ions during incubation in the medium containing that radioactive ion (Prush and Dunham 1972). Such great discrepancies in observations of the same phenomenon in protozoan cells are difficult to explain. It is possible that the observed differences in the Cl^- distribution across membrane even between related organisms as *Amoeba proteus* and *Chaos chaos* cells are due to basic differences between those species or they could result from the various experimental techniques used to evaluate the ionic distribution.

The detailed speculations on regulatory mechanism of chloride distribution in *Blepharisma* or other protozoans are premature at present state of knowledge. The experiments properly directed, utilizing the Cl^--selective glass microelectrodes for measuring the ionic activity of chloride in vivo, as well as other selective to other ions exchangers should provide further experimental facts for elucidation of chloride distribution and its relation to the bahaviour of protozoan cells.

ACKNOWLEDGEMENTS

We would like to thank Dr. Jerzy Sikora for his helpful suggestions on this manuscripts.

REFERENCES

Aickin C. C. and Brading A. F. 1982: Measurement of intracellular chloride in guinea-pig was deferens by ion analysis, ^{36}Cl efflux and microelectrodes. J. Physiol. (London), 326, 139–154.

Akita Y. K. 1941: Electrolytes in *Paramecium*. Zoology 12, 99–119.

Baumgarten C. M. 1981: A program for calculation of activity coefficient at selected concentrations and temperatures. Comput. Biol. Med., 11, 189–196.

Bolton T. B. and Vaughan-Jones R. D. 1976: The use of electrodes containing liquid ion-sensitive resin to measure the intracellular chloride activity of skeletal muscle. J. Physiol., (London), 260, 6–7.

Bruce D. L. and Marshall J. M. 1965: Some ionic and bioelectric properties of the amoeba *Chaos chaos*. J. Gen. Physiol., 49, 151–178.

Caille J. P., Ruiz-Ceretti E. and Schanne O. F. 1981: Intracellular chloride activity in rabbit papillary muscle: effect of ouabain. Am. J. Physiol., 240, 183–188.

Dean J. A. 1985: Lange's Handbook of Chemistry. Mc Graw-Hill, New York, 1–187.

http://rcin.org.pl

Dunham P. B. and Child F. M. 1961: Ion regulation in *Tetrahymena*. Biol. Bull. 121, 129–140.
Dunham P. B. 1964: The adaptation of *Tetrahymena* to high NaCl environment. Biol. Bull., 126, 373–390.
Dunham P. B. and Stoner L. C. 1967: Regulation of intracellular sodium concentration by the contractile vacuole in *Tetrahymena* J. Protozool. 14, Suppl., 345.
Kerkut G. A. and Meech R. W. 1966: Microelectrode determination of the intracellular chloride concentration in nerve cells. Life Sci., 5, 453–456.
Kernan R. P., MacDermott M. Wesphal W. 1974: Measurement of chloride activity within frog sartorius muscle fibers by means of chloride--sensitive microelectrodes. J. Physiol. (London), 249, 60–61.
Keynes R. D. 1963: Chloride in the squid giant axon. J. Physiol. (London), 169, 690–705.
Kunze D. L. and Brown A. M. 1971: Internal potassium and chloride activities and the effects of acetylcholine on identifiable *Aplysia* neurons. Nature New Biol., 229, 229–231.
Meier P. C., Lanter F., Ammann D. Steiner R. A. and Simon W. 1982: Applicability of available ion-selective liquid-membrane microelectrodes to intracellular ion-activity measurements. Pflugers Archiv. Ges. Physiol., 393, 23–30.
Moreton R. D. and Gardner D. R. 1981: Increased intracellular chloride activity produced by the molluscicide N-(triphenylmethyl)morpholine (Frescon), in *Lymnaea stagnalis* neurons. Pestic. Biochem Physiol., 15, 1–9.
Prush R. D. and Dunham P. B. 1972: Ionic distribution in *Amoeba proteus*. J. Exptl Biol., 56, 551–563.
Russel J. M. and Brown A. M. 1972: Active transport of chloride by the giant neuron of the *Aplysia abdomina* ganglion. J. Gen. Physiol., 60, 499–518.
Russel J. M. 1978: Effects of ammonium and bi-carbonate-CO_2 on intracellular chloride levels in *Aplysia* neurons. Biophys. J. 22, 131–138.
Wood D. C. 1982: Membrane permeabilities determining resting, action and mechanoreceptor potentials in *Stentor coeruleus*. J. Comp. Physiol. 146, 537–550.
Vaughan-Jones R. D. 1979: Non-passive chloride distribution in mammalian heart muscle: microelectrode measurement of the intracellular chloride activity. J. Physiol. (London) 295, 83–109.
Ziskind L. Kunze D., Brown A. M. and Werman R. 1975: Internal chloride and potassium activities in snail neurons. Israel J. Med. Sci., 11, 71–72.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 273-283 (1989)

Factors Influencing the Development of Population of the Rumen Ciliates *Anoplodinium denticulatum* in Vitro

Tadeusz MICHAŁOWSKI, Irena LANDA and Piotr MUSZYŃSKI

Department of Vertebrate Animal Physiology, Zoological Institute, Warsaw University, 93 Żwirki i Wigury Str., 02-089 Warszawa, Poland

Received on 24th October, 1988

Synopsis. The rumen ciliates *Anoplodinium denticulatum* were grown in vitro. The ciliates were able to survive for several months in a medium consisting of a salt solution based on phosphates an powdered meadow hay. When hay was supplied at the ratio of 0.8-1.0 mg/ml culture/day the population density was over 2000 cells/ml. The influence of wheat gluten, casein and urea on the cultured protozoa was related to the age of the cultures. These substances had no effect on, or decreased, the number of ciliates during the first 3-4 months of the culture but were necessary for maintenance of older populations. Urea in doses of 0.18 mg/ml culture/day or more was toxic for the cultured protozoa. Pectins, and especially pure barley starch, decreased, and cellulose powder increased, the concentration of *Anoplodinium denticulatum* in vitro. The ciliates were also able to survive in a medium consisting of culture salt solution, cellulose and wheat gluten when it was supplemented with stabilized rumen extract. The results of microscopical observations of the intracellular digestion of hay particles, cellulose and starch are described.

The importance of rumen microorganisms lies in the liberation of energy from a matter indigestible for ruminants and on the synthesis of microbial protein which is the main source of amino acids for the host animal. The participation of particular species of bacteria and protozoa in the rumen metabolism is rather poorly recognized and requires more information concerning the biology and especially the physiology of nutrition of these microorganisms. The study of their biology under natural conditions is complicated by the interactions which exist between

http://rcin.org.pl

different species. Thus more simple conditions are necessary for such investigations, i.e. in vitro conditions which make possible the cultivation of single-species populations of rumen microorganisms. In our earlier papers we described the observations concerning the factors influencing the growth of two common species of rumen ciliates i.e. *Entodinium exiguum* (Michałowski et. al. 1985) and *Diploplostron affine* (Michałowski et al. 1986a). In the present paper, our observations concerning the growth of *Anoplodinium denticulatum* are described.

Material and Methods

The protozoa used for initiation of the cultures originated from the rumen of cattle. Portions of fresh rumen fluid (1 ml) were inoculated to the Erlenmeyer flasks containing 39 ml of culture medium. This culture medium consisted of salt solution and food. The "caudatum type" salt solution (Coleman et al. 1972) was used as the liquid part of the medium while the food was a mixture of hay (60%), barley flour (24%) and wheat gluten (16%). Cultivation was performed by methods routinely used in our laboratory. The ciliates were fed every day and every fourth day they were transferred into fresh medium according to Michałowski (1975). The ciliate species which survived were different *Entodinia, Anoplodinium denticulatum, Eudiplodinium maggii* and *Epidinium ecaudatum*. Single-species cultures of *Anoplodinium denticulatum* were prepared by picking typical forms of these ciliates with a micropipette and than developing their populations. As many as 100–200 cells of these protozoa were introduced into a flask containing 2–3 ml of the culture salt solution. Fresh portions of this solution of 1–2 ml volume were added daily to all isolates during the first week of cultivation. Small amounts of food were also given. When the concentration of ciliates reached 100–200 cells/ml, the cultures were transferred to other flasks and cultivated routinely as described above. When the concentration increased to about 1000 cells/ml they were transferred into the flasks of the apparatus for continuous culture of rumen ciliates (Michałowski, 1979) and were cultivated for many months. Samples of these protozoa were used for inoculation of the experimental cultures.

Four types of salt solution were used for preparation of the culture media (Table 1). Other components of the culture media were: ground meadow hay, barley flour, pure barley starch, pectins, cellulose powder (Koch Light Lab.), wheat gluten, casein (BDH) and urea (Polskie Odczynniki Chemiczne). All these substances were used for preparation of the food. They were mixed in the proportions related to a particular experiment. Stabilized rumen ekstract was also used in one experiment. These substances which were not available commercially, were prepared in the laboratory. Pure barley starch was prepared according to Whelan (1955) and whet gluten according to Klein (1933) and Pace (1955). The stabilized rumen extract was supplied by Dr. A. Ziołecki (Institute of Animal Physiology and Nutrition, Pol. Acad. Sci., Jabłonna near Warsaw). The content of α amino N was measured as described by Muszyński et al. (1985).

The experimental cultures were initiated by inoculation of the suspension of *Anoplodinium denticulatum* (20 ml) into flasks containing 20 ml of culture salt

http://rcin.org.pl

solution and an appropriate food. The cultivation procedure was the same as described above. The transferring of the ciliates to fresh medium with a frequency of 1, 2 and 3 days was, however, also used. Three cultures of ciliates were always run simultaneously in relation to any factor studied.

The samples for counts of protozoa were taken on the transfer days. They were fixed with an equal volume of 4% formaldehyde solution. The number of protozoa in the samples were estimated under the light microscope according to Michałowski (1975). The bacteria were counted with the use of Thoma counting chamber. Each sample was counted three times.

Table 1

The chemical composition of salt solutions used for cultivation of *Anoplodinium denticulatum* (g/l)

Ingredient	A	B	C	D
K_2HPO_4	6.3	4.9	0.0	0.0
KH_2PO_4	5.0	3.8	1.0	0.0
$Na_2HPO_4 \times 12H_2O$	0.0	0.0	0.0	0.0
$NaHCO_3$	0.0	6.6	5.0	9.8
KCl	0.0	0.0	0.0	0.56
NaCl	0.65	0.49	6.0	0.47
$CaCl_2 \times 6H_2O$	0.09	0.07	0.2	0.08
$MgCl_2$	0.0	0.0	0.0	0.06
$MgSP_4 \times 7H_2O$	0.09	0.07	0.2	0.0
CH_3COONa	0.75	0.0	0.0	0.0
pH	6.84	7.53	7.76	8.52

A — "caudatum type" salt solution, B — "simplex type" salt solution (both according to Coleman et al. 1972). C — "Hungate type" salt solution (according to Hungate, 1942), D — artificial saliva (after McDougal, 1948)

The intake and digestion of hay particles, cellulose and starch was examined microscopially after starvation of ciliates for 36 h. Portions of starved cultures (40 ml) were introduced to the incubation flasks and powdered hay or cellulose powder or starch (40 mg) together with wheat gluten (5 mg) were then added. The content of the flasks was saturated with CO_2 for 3 min (CO_2 flow intensity of 50–60 cm^3/min). The flasks were then stoppered with a rubber stopper and incubated for 48 h at 38°C. Samples of the incubated material were taken at 1, 3, 6, 24 and 48 h after start of incubation. Samples of starved protozoa were also taken. The sampled material was fixed with 4% formaldehyde solution as described above. The experiment was repeated three times. Collected samples were analysed by determination the proportion of the ciliates containing particles of the offered substances inside their cells. Each sample was analysed three times.

The results obtained were statistically analysed using Student's t-test. The correlation coefficient between ciliate concentration and proportion of some diet components was also calculated. The statistical analysis were made according to Ruszczyc (1970).

http://rcin.org.pl

Results

Transferring of *Anoplodinium denticulatum* to fresh medium with a daily frequency caused disappearance of ciliates within 5–6 days. They survived, however, for a long period when transferred every 2, 3 or 4 days. Therefore the experimental cultures of these protozoa were transferred every 4 days similar to the cultivation procedure of other species cultured tat the same time in the laboratory.

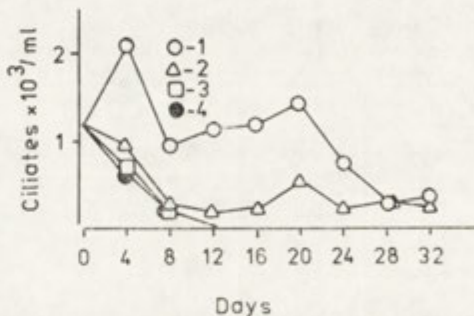


Fig. 1. The changes in number of *Anoplodinium denticulatum* cultured in the medium based on "caudatum type" (1), "Hungate type" (2), "simplex type" (3), or artificial saliva (4). All cultures received daily a 40 mg mixture of hay (80%), barley flour (3.5%) and wheat gluten (16.5%)

Of the culture salt solutions tested here only "caudatum type" salt solution and "Hungate type" salt solution provided appropriate conditions for survival of the ciliates, *Anoplodinium denticulatum*, in vitro. The concentration, however, of these protozoa was higher when "caudatum type" solution was used (Fig. 1). The mean number of bacteria in these cultures was 8.2×10^7 and 8.9×10^7/ml respectively for "caudatum" and "Hungate type" salt solution.

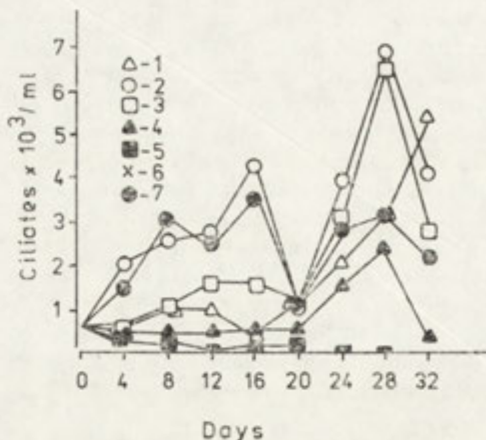


Fig. 2. The changes in concentration of *Anoplodinium denticulatum* in the cultures fed on a mixture of different proportion of hay and barley flour. The daily ration of food was a 40 mg/culture. The content of hay was 83.5 (1), 80 (2), 60 (3), 40 (4), 20 (5) and 0% (6) of the daily ration. The content of barley flour was 0, 3.5, 23.5, 43.5, 63.5 and 83.5% respectively. Wheat gluten formed 16.5% of the daily ration. (7) — cultures receiving daily 40 mg of hay

http://rcin.org.pl

The density of the population of *Anoplodinium denticulatum* in relation to the proportion of hay and barley flour in the diet is presented in Fig. 2. There were large variations in ciliate number during the cultivation period. In general a negative correletion was found between proportion of barley flour in the diet and population density of *Anoplodinium denticulatum* ($r = -0.96$; $P < 0.01$).

Table 2

The population density of *Anoplodinium denticulatum* ($\times 10^3$/ml) and bacteria ($\times 10^7$/ml) in the cultures receiving different diets. Mean values $\pm$ S.D.

Diet	Ciliates	Bacteria
Hay	3.0±0.65	7.2±1.59
Hay + wheat gluten	2.9±0.47	7.4±1.13
Hay + casein	2.5±0.41	7.7±0.91
Hay + urea	2.4±0.74	7.1±1.19
Hay + barley flour	2.9±0.56	8.2±1.59
Hay + ,, ,, + wheat gluten	1.8±0.49	8.1±2.84
Hay + ,, ,, + casein	1.8±0.42	8.9±2.24
Hay + ,, ,, + urea	1.7±0.50	7.9±1.43

Hay was supplied in the ratio of 0.8; barley flour — 0.035; wheat gluten — 0.165; casein — 0.15 and urea — 0.065 mg/ml/day

The ciliates, *Anoplodinium denticulatum*, were able to survive in the medium containing powdered hay as a sole source of nutrients for cultured microorganisms. This possibility was observed for the first 3–4 months ofter isolation of the ciliates from the mixed populations of rumen protozoa. When hay was supplied at the ratio of 0.8–1.0 mg/ml culture/day the number of ciliates was over 2000 cells/ml. The supply of wheat gluten, casein or urea to the medium consisting of "caudatum type" salt solution and hay (Table 2) had no effect on the number of microorga-

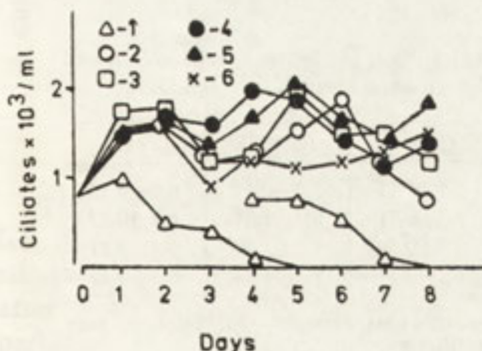


Fig. 3. The changes in concentration of *Anoplodinium denticulatum* receiving daily 32 mg of hay (1) supplemented with 1.65 (2), 3.3 (3), 6.6 (4), 8.25 (5), and 9.9 (6) mg of wheat gluten. The volume of all cultures was 40 ml

http://rcin.org.pl

nisms (P>0.05). The supplement of the same nitrogen bands to the medium containing a mixture of hay and barley flour caused a decrease in the number of protozoa by about 35–55% (P<0.01). The doses of wheat gluten, casein and urea were isonitrogenous and provided about 1.2 mg of α amino N/culture flask/day.

The supplementation of the culture medium with nitrogen was necessary for maintenance of ciliates in vitro in the experiments performed 6 or more months after isolation of *Anoplodinium denticulatum*. The positive effect of the supplement of wheat gluten Fig. 3 as well as urea (Fig. 4) to the medium containing of "caudatum type" salt solution and

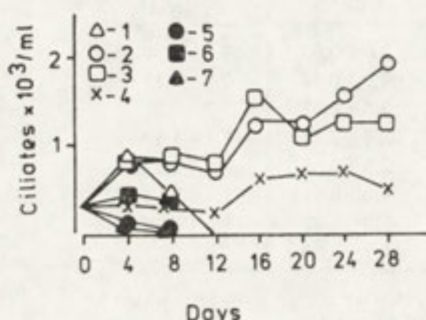


Fig. 4. The changes in number of *Anoplodinium denticulatum* receiving daily 32 mg of hay (1), supplemented with 1.2 (2), 2.4 (3), 3.6 (4), 7.2 (5), 9.6 (6) and 14.4 (7) mg of urea. The volume of all cultures was 40 ml

hay was observed. The doses of wheat gluten offered formed from 4.9 to 23% of the daily ration of food. The mean number of ciliates tended to increase with an increase of the protein dose up to 20.6% of wheat gluten in the ration ($r = 0.95$; $P < 0.05$). These mean values were: 1330 ± 348, 1510 ± 313, 1580 ± 285 and 1680 ± 222 cells/ml. The mean number of ciliates in the cultures supplemented with the highest dose of wheat gluten was, however, only 1240 ± 329 cells/ml. The ciliates were able to survive in the medium supplemented with urea at the ratio of

Table 3

The number of *Anoplodinium denticulatum* ($\times 10^3$/ml) and bacteria ($\times 10^7$/ml) as well as pH values in the cultures receving different diets. Mean values ± S.D.

Diet	Ciliates	Bacteria	pH
Hay + wheat gluten	2.1±0.15	5.8±0.81	6.6±0.22
Hay + wheat gluten + cellulose	3.3±0.71	6.7±1.21	6.7±0.27
Hay + wheat gluten + starch	0.8±0.54	7.2±1.28	6.4±0.31
Hay + wheat gluten + pectins	1.4±0.17	8.3±1.14	6.5±0.23

Hay was supplied in the ratio of 0.56; wheat gluten — 0.1; celulose, pure barley starch and pectins — 0.25 mg/ml culture/day

http://rcin.org.pl

0.03, 0.06 and 0.09 mg/ml culture/day. The higher doses of urea i.e. 0.18, 0.225 and 0.36 mg/ml culture/day caused the death of the ciliates within 12–20 days. The mean concentration of bacteria in both types of cultures varied between 7.7×10^7 and 8.4×10^7/ml ($P > 0.05$).

The concentration of *Anoplodinium denticulatum* in the medium supplemented with different polysaccharides is presented in Table 3. The ciliates died within 20 days when wheat gluten was omitted. The supplementation of the culture medium consisting of "caudatum type" salt solution, hay and wheat gluten with cellulose caused an increase in ciliate number by about 57% ($P < 0.01$). The supplement of starch and pectins caused a decrease in population density of *Anoplodinium denticulatum* by about 62 and 33 % respectively ($P < 0.01$). On the other hand the increase in the hay dose from 0.65 to 0.9 mg/ml culture/day caused an increase in the population density of the cultured protozoa from 2100 to 2600 cells/ml ($P \leq 0.05$). A statistically significant increase in the number of bacteria was observed only in the case of supplementation of the culture medium with pectins ($P < 0.01$). No significant differences in pH value were found ($P > 0.05$), the titer, however, in the cultures supplemented with pure barley starch was somewhat lower than in the control cultures or the cultures supplemented with cellulose.

The cultured ciliates were also able to survive in a medium consisting of culture salt solution, cellulose powder and wheat gluten when this medium was supplemented with stabilized rumen extract (Fig. 5). The ciliates receiving hay only or pure cellulose and wheat gluten died within 12–20 days.

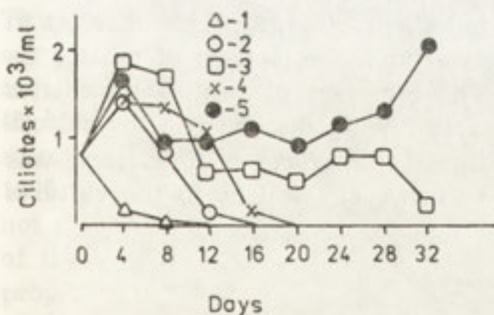


Fig. 5. The changes in concentration of *Anoplodinium denticulatum* receiving daily 34 mg of cellulose (1) supplemented with 6 mg of wheat gluten (2), or 6 mg of wheat gluten and 5 mg of stabilized rumen extract (3). (4) — the cultures receiving daily 34 mg of hay. (5) — the cultures receiving daily 34 mg of hay and 6 mg of wheat gluten. The volume of all cultures was 40 ml

The ciliates were observed to readily ingest hay and cellulose particles. Ingestion of starch grains was also observed. The proportions of ciliates with these three substances inside the cells at different time after feeding are presented in Table 4. The percentage of ciliates with particles of hay, cellulose and starch increased during the first 6 h after

http://rcin.org.pl

Table 4

Percentage of this ciliates with hay particles, cellulose or starch grains inside the cells at different times (h) after giving these substances to protozoa. Mean values ± S.D.

Time after feeding	Hay	Cellulose	Starch
1	61.3±7.58	60.5±9.31	32.3±7.77
3	84.3±3.72	79.7±9.97	59.7±8.02
6	89.2±3.43	92.0±2.00	69.7±2.08
24	73.7±11.60	86.7±8.24	41.3±4.62
48	38.5±6.80	57.2±14.69	4.3±0.58

feeding and was followed by a gradual decrease. It was apparent that the loss of the cellulose particles from the cells occurred more slowly than the loss of hay or starch grains. At 48 h after giving cellulose to the ciliates, as many as 57% of the protozoa where showed particles of this polysaccharide in their endoplasmic sacs. Some of them were observed at this time with small grains of the storage carbohydrate in the ectoplasm. About 39% of the total ciliates observed had ingested hay particles at 48 h after feeding. In the majority of cases these were only very small and single particles. Grains of storage polysaccharide were not observed at this time in the ciliates fed with hay as well as with starch. The observations made showed that ciliates, *Anoplodinium denticulatum*, are ingesting large quantities of cellulose and hay particles. The majority of cells were observed to be completely filled with these two substances at 6 h after giving the food to the protozoa. Such was not observed in the case of starch ingestion. Storage polysaccharides in the form of small grains were observed in the ectoplasm of ciliates ingesting cellulose, hay and starch. The ingestion of pectins by ciliates was not observed since these substances rapidly dissolved in the culture medium. A rapid development of thread-like colonies of bacteria was found during incubation of these cultures as well as in the cultures incubated with starch.

Discussion

As early as 1961 Grądzka-Majewska cultured *Anoplodinium denticulatum* in a medium consisting of filtered and sterilized rumen fluid and ground leaves of certain plants. The ciliates of this species were cultured by Coleman et al. (1976) who considered them to be *Diplodinium*

http://rcin.org.pl

monacanthum and *Diplodinium pentacanthum*. According to Dogiel (1927) these both species are froms of *Anoplodinium denticulatum*.

Coleman et al. (1976) used "simplex type" salt solution for cultivation of these protozoa. It was, however, supplemented with fresh rumen fluid. Rumen fluid was not necessary in our cultures when "caudatum type" salt solution was used while the cultivation in "simplex" medium was unsuccesfull.

When *Diplodinium pentacanthum* was grown from small inocula the mean generation time of the population was 8.5 h (Coleman et al. 1976). In general the doubling time of the populations in our cultures was longer than 24 h. However, an increase in ciliate number by over 100 % at 24 h was observed from time to time.

There were different reactions on supplementation of the culture medium with protein as well as non-protein nitrogen related to the age of the ciliate cultures. The causes of these differences are unknown. It is possible that the changes in some properties of the components of culture medium took place. The changes in ciliate requirements, however, cannot be excluded.

The effect of urea on the ciliates, *Anoplodinium denticulatum*, depended on the age of the cultures and the dose of this substance. The doses over 0.18 mg/ml culture/day were toxic for these protozoa. Thus these findings are similar to our earlier observations concerning *Entodinium caudatum* (Michałowski et. al. 1987).

The reaction of *Anoplodinium denticulatum* on supplementation of the culture medium with cellulose, pectins and starch was similar to the reaction of *Diploplastron affine* (Michałowski et al. 1986 a). This species, however, seems to posses a different feeding behavior as compared to *Entodinium caudatum*. Ciliates, *Anoplodinium denticulatum*, were observed ingesting the food particles over a longer period (6 h) than *Entodinium caudatum* which engulfed practically all the starch grains within the first hour after feeding (Michałowski et al. 1986 b). As with *Diploplastron affine*, *Anoplodinium denticulatum* did not react by increase in its number in relation to increase of the amount of the starch-rich component in the diet. Moreover, an increase in the proportion of barley starch in the diet caused a decrease in the number of these ciliates. On the other hand, however, starch was degraded more rapidly inside the ciliate cells than cellulose or hay (Table 4). It was also observed that the majority of the starch grains remained in the medium. They were very quickly attacked by bacteria and as a consequence of this action there was a very rapid growth of thread-like colonies of these organisms. Perhaps the restricted growth of *Anoplodinium denticulatum* observed in the medium supplemented with starch or

http://rcin.org.pl

barley flour was the consequence of unfavourable changes in the properties of the environment due to abnormal development of these bacteria and liberation of some products from the rapidly degraded starch by bacteria as well as by the protozoa themselves. One of these changes could be an increase in the acidity of the medium (Table 3).

The ciliates, *Anoplodinium denticulatum*, were observed ingesting and degrading the particles of cellulose and hay. They were also able to survive in a medium consisting of culture salt solution and hay or culture salt solution, cellulose powder and wheat gluten supplemented with stabilized rumen extract. These observations suggest a role of these ciliates in fibre digestion in the rumen.

Pure cellulose was more slowly digested than hay particles (Table 4). It is possible that the technical preparation of the cellulose had made it more resistant to the action of cellulolytic enzymes as compared with an, untreated, natural form of this polysaccharide from the hay particles.

The study was carried out under a project coordinated by the Institute of Animal Physiology and Nutrition, Jabłonna near Warsaw.

REFERENCES

Coleman G. S., Davies J. I. and Cash M. A. 1972: The cultivation of *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum* in vitro. J. Gen Microbiol., 73, 509–521.

Coleman G. S., Laurie J. I., Bailey J. E. and Holdgate S. A. 1976: The cultivation of cellulolytic protozoa isolated from the rumen. J. Gen. Microbiol., 95, 144–150.

Dogiel V. A. 1927: Monographie der Familie *Ophryoscolecidae*, Arch. Protistenkd., 59, 1–288.

Grądzka-Majewska I. 1961: The ability of *Anoplodinium denticulatum* Dogiel to utilize some plants. Acta Parasit. Pol. 9, 169–191.

Hungate R. E. 1942: The culture of *Eudiplodinium neglectum* with experiments on the digestion of cellulose. Biol. Bull., 83, 303–319.

Klein G. 1933: Handbuch der Pflanzenanalyse. Springer Verlag, Wien, pp. 333–354.

McDougal E. J. 1948: Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J., 43, 99–109.

Michałowski T. 1975: The effect of certain feedingstuffs on rumen ciliate protozoa in vitro. J. Agric. Sci. Camb., 85 151–158.

Michałowski T. 1979: A simple system for continuous culture of rumen ciliates. Bull. Acad. Pol. Sci. Cl. II Sér. Sci. Biol., 27, 581–583.

Michałowski T., Szczepkowski P. and Muszyński P. 1985: The factors affecting the cultivation of the rumen ciliate protozoon *Entodinium exiguum* in vitro. Acta Protozool., 24, 297–305.

Michałowski T., Szczepkowski P. and Muszyński P. 1986 a: The nutritive factors affecting the growth of the rumen ciliate *Diploplastron affine* in vitro. Acta Protozool., 25, 419–426.

Michałowski T., Szczepkowski P. and Muszyński P. 1986 b: The effect of starch, size of starch grains and proportion of amylose and amylopectin in the diet on the growth of *Entodinium caudatum* in vitro. Protistologica, 22, 175–180.

http://rcin.org.pl

Michałowski T., Landa I., Muszyński P. and Szczepkowski P. 1987: The influence of non-protein-nitrogen on the growth of rumen ciliate *Entodinium caudatum* in vitro. Acta Protozool., 26, 329–334.
Muszyński P., Michałowski T. and Szczepkowski P. 1985: The influence of protein on the number of rumen ciliates *Entodinium caudatum* and *Diploplastron affine* in vitro. Acta Protozool., 24, 307–317.
Pace J. 1955: Seed proteins. In: Modern Methods of Plant Analysis, (eds. K. Peach and M. V. Tracey), Springer Verlag, Berlin, Göttingen, Heidelberg, 4, 69–105.
Ruszczyc Z. 1970: Metodyka Doświadczeń Zootechnicznych. PWRiL Warszawa.
Whelan W. J. 1955: Starch, glycogen, fructosans and similar polysaccharides. In: Modern Methods of Plant Analysis, (eds. K. Peach and M. V. Tracey), Springer Verlag, Berlin, Göttingen, Heidelberg, 2, 145–196.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 285-291 (1989)

Epizootics of Reptilian Amoebiasis at the Zoological Gardens of Poznań (Poland); Epidemiological and Chemotaxonomic Studies

Witold KASPRZAK[1], Waldemar GOLEC[2] and Edward HADAŚ[1]

[1] Department of Biology and Medical Parasitology, Academy of Medicine, 10 Fredry str., 61-701 Poznań; [2] Poznań Zoological Gardens, 19 Zwierzyniecka str., 60-814 Poznań, Poland

Received on 21st November, 1988

Synopsis. Since 1968 fatal epizootic dysentery has been recurrently observed in snakes (*Eunectes notaeus, Cyclagras gigas, Boa constrictor occidentalis, Epicrates cechris, Coluber jugularis caspius, Bothrops neuviedi meridionalis*) at the Poznań Zoological Gardens. The occurrence of amoebae in stools, intestinal wall and liver and the clinical course of the disease suggested that the cause of the epidemics was *Entamoeba invadens*. The snakes, were kept together with turtles (*Pseudomys ornata callirostis, Pseudomys scripta elegans*), which were also infected with amoebae. The study attempted to prove the source of infection in snakes by experimental infections of grass snakes (*Natrix natrix*) with amoebae isolated from turtle and by comparing the protein patterns of amoebal isolates both from snakes and turtles. Experimental infection proved the amoebae to be pathogenic. The disc electrophoresis of soluble proteins of all strains isolated from snakes and turtles were identical or very similar to the protein patterns of the control well established strains of *E. invadens* (Prague and BAH). On the other hand, all the isolates showed remarkable differences in their protein make-up when compared with control *E. tarrapinae* (strain EDP). This observation showed that the source of epizootic amoebiasis of snakes were infected turtles, and that the disc electrophoresis of amoebal proteins clearly differentiated pathogenic *E. invadens* from non-pathogenic *E. terrapinae*.

Since 1968 fatal cases of dysentery in snakes have been recurrently observed at the Poznań Zoological Gardens (Golec and Kasprzak 1977). The disease, which was considered epizootic, concerned the following species of animals: yellow anaconda (*Eunectes notaeus*), Boipevussu

http://rcin.org.pl

snake (*Cyclagras gigas*), western boa (*Boa constrictor occidentalis*), rainbow boa (*Epicrates cechris crassus*), caspian arrow-snake (*Coluber jugularis caspius*), and *Bothrops neuviedi meridionalis*.

The occurence of numerous amoebae in dysenteric stools and — although small in numbers — in the intestinal wall as well as the clinical course of the disease, suggested that the amoebae belonged to *Entamoeba invadens* and were the cause of the epidemics. It is assumed that the infection of *E. invadens* was brought with the specimens of *Eunectes notaeus* imported in 1968. The clinical symptoms of the disease and pathological findings were the same as given by other authors and were described elsewere (Golec and Kasprzak 1977). Administration of metronidazole and macrocycline failed in most cases. To prevent the spread of the disease, ill animals and those suspected to be infected were isolated, the vivaria disinfected and the bedding changed. In spite of the preventing measures the epizootics have occurred recurrently.

The snakes were kept together with turtles (*Pseudomys scripta elegans* and *P. ornata callirostis*) in the same vivaria. Because the infection of the turtles with amoebae of *Entamoeba* genus was proved by microscopic examinations of the faeces (wet and permanent — Schaudin's fixative, Heidenhain's iron-hematoxylin method of staining — preparations), the source of persistent infections in the snakes seemed to have been the infected turtles. However, turtles can be infected by two morphologically indistinquishable species of amoebae, forming 4-nucleate cyst, *E. terrapinae* and *E. invadens*. In cases, where morphological criteria do not differentiate between protozoan species, the protein patterns can be used as a criterion.

In concjunction with these findings this study attempted to prove the source of the infection of snakes by experimental infection with amoebae isolated from a turtle and by comparing the protein patterns of amoebal isolates which were isolated both from snakes and from turtles.

Material and Methods

Experimental infection

The study was carried out on *Entamoeba* sp. (isolate POC-31) isolated from a turtle (*Pseudomys ornata callirostis*) occupying the same vivarium with snakes that died of dysentery. In a parallel control experiment the BAH strain of *E. invadens* was used. The xenic isolates of amoebae were cultivated at 24°C on liquid medium after Pavlova (1938) and subcultured once a week. Inocula for experimental infection were prepared from 3–4-day-old cultures by centrifugation at 900 g for 5 min followed by threefold washing in physiological salt solution. The

http://rcin.org.pl

volume of the inocula varied within 0.2 to 0.3 ml and the count of amoebae was about $5 \times 10^4 - 1 \times 10^5$.

For experimental infection with amoebae three grass snakes (*Natrix natrix*) were used. As control experiment two grass snakes were infected with *E. invadens* strain BAH. The animals were captured in the vicinity of Poznań and kept individualy in separate vivaria at room temperature (20° to 25°C). Repeated faecal examinations showed no spontaneous infection with amoebae. To inoculate the snakes with amoebae, stomach tube was used. The dead snakes were authopsied, and the gross condition of the organs was noted. Portions of large intestine and liver were inoculated on Pavlova's medium to establish culture of amoebae and were also fixed for pathomorphological examination.

Disc electrophoresis of amoebal proteins

To study the protein make-up, the following xenic isolates of amoebae were used: EN-2 from *Eunectes notaeus*, CG-23 from *Cyclagras gigas*, PSE-29 from *Pseudomys scripta elegans*, POC-31 from *Pseudomys ornata callirostis*, and POC-31/Nn 2 from *Natrix natrix* infected experimentally with POC-31 isolate. As control, two strains of *E. invadens* (Prague and BAH) and the EDP strain of *E. terrapinae* were investigated *. The amoebae were cultured in a liquid medium after Pavlova (1938) at 24°C, and were harvested from 7-day-old cultures by centrifugation at 900 g for 5 min followed by threefold washing in physiological salt solution.

Homogenates were prepared by threefold freezing and thawing of the washed and centrifuged amoebae. The homogenates were mixed with 0.5 M tris-glycine buffer pH 8.5 (with 5% potassium sulfate and 0.5% 2-mercapto-ethanol added) in a 1 : 3 ratio for 6 hs at 0°C, and centrifuged at 20000 g for 30 min to extract the soluble proteins. Protein content of samples was estimated by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Disc electrophoresis was performed on 7.5% polyacrylamide gel and carried out in a Quickfit apparatus according to the method of Davis and Ornstein (1959) by omitting the spacer gel. As electrode and separation gel buffer a tris-glycin buffer pH 8.5 was used. After one hour's preelectrophoresis the samples containing 150–200 µg of protein were placed on the top of the gel together with bromophenol blue marker and sucrose. The electrophoresis was performed at 4°C for 60 min at 2.5 mA for each gel. The gels were fixed, stained with 0.1% Amido Black 10B in 7.5% acetic acid with 5% ethanol for 30 min, and destained electrophoretically in 5% acetic acid at 190 mA for 20–30 min.

The protein patterns were recovered densitometrically in a Vitatron densitometer with a filter of a maximum transmittance at 576 µm. The taxonomic assessement was based entirely on Rf values, and the percentage similarity between the two taxa was calculated according to Whitney et al. (1969). The bands were given the same Rf values when there was more than a 50% overlap. The band density was not taken into consideration.

---

* The Prague strain was initially provided by Professor O. Jirovec (Czechoslovak Academy of Sciences), and the BAH and EDP axenic strains were supplied by Dr. R. A. Neal (The Wellcome Research Laboratories, Great Britain).

http://rcin.org.pl

Results

Experimental infection

All the grass snakes infected experimentally with amoeba POC-31 isolate from a turtle *Pseudomys ornata callirostis* or with control BAH strain of *E. invadens* developed amoebiasis within 1–2 weeks post-infection and died after 2–3 weeks post-infection. At autopsy the large intestine of all animals was thickened and ulcerated. Abscesses were found also in the liver. Few amoebae were observed in fresh wet and permanent smears of scrapings from the ulcerated intestine-wall. One isolate was successfully established in culture by inoculation of material from ulcerated intestine. This isolate (POC-31/Nn2) was compared with other strains in electrophoretic study.

Disc electrophoresis of amoebal proteins

Qualitative electrophoretic analysis of amoebal soluble proteins gives distinct and reproducible patterns characteristic for the two *Entamoeba* species. The 17 bands which could be taken into consideration in differentiation of *E. invadens* and *E. terrapinae* are shown diagramatically in Fig. 1. Table 1 shows the percentage similarity of the protein bands between all combinations of the amoeba isolates investigated.

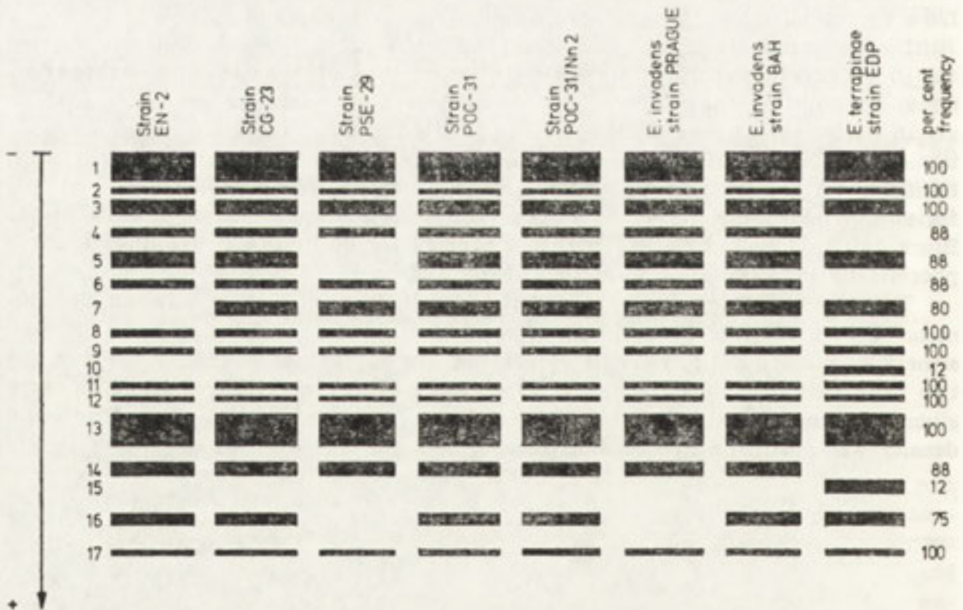


Fig. 1. Distribution of protein bands in *Entamoeba* strains as expressed by Rf values (numbered from 1 to 17) and per cent frequency

http://rcin.org.pl

Table 1

Percentage similarity of protein bands between all combinations of examined strains based on the distribution of the bands

	EN-2	CG-23	PSE-29	POC-31	POC-31/Nn2	Prague	BAH	EDP
EN-2	100	93	80	93	93	87	93	65
CG-23		100	87	100	100	93	100	71
PSE-29			100	87	87	93	87	59
POC-31				100	100	93	100	70
POC-31/Nn2					100	93	100	70
Prague						100	93	63
BAH							100	70
EDP								100

All the isolates of amoebae both from snakes and from turtles show total or a very high percentage similarity (from 87 to 100%) to the two control strains of *E. invadens* (Prague and BAH). On the other hand, all the strains differ in their protein patterns from the control EDP strain of *E. terrapinae* (the percentage similarity ranged between 59 and 71). The lack of a total percentage similarity (93%) of protein bands between the two well established laboratory strains of *E. invadens* (Prague and BAH) should be emphasized.

Discussion

Infection of reptiles with *E. invadens* in zoological parks is recognized as apizootic with major loss of animals (Hill and Neal 1954; Steck 1961; Donaldson et al. 1975; Jacobson et al. 1983). Meerovitch (1961) considered that highly pathogenic *E. invadens* does not infect snakes in natural surroundings, however, the infection frequently occurs in captivity through water and utensil contamination. Evidences have also proved that the turtles and tortoises act as natural hosts of *E. invadens* (Meerovitch 1958; Donaldson et al. 1975; Mishra and Gonzales 1978; Jacobson et al. 1983). Because the infection of turtles with amoebae is asymptomatic, they remain a potential source of infection to the snake colonies.

However, turtles could be parasitized by two morphologically indistinguishable amoebae forming 4-nucleate cysts, pathogenic *E. invadens* and non-pathogenic *E. terrapinae*. Said-Fernández and López-Revilla (1976) showed the usefulness of electrophoretic analysis of proteins in

http://rcin.org.pl

differentiating between species of *Entamoeba*. The soluble protein patterns of all isolates of amoebae from snakes with amoebiasis and from turtles in our study were identical or very similar to the protein patterns of both control strains of *E. invadens*. Also the amoebae reisolated from experimentally infected grass snake shows the same protein make-up. On the other hand, all the isolates showed remarkable differences in their protein patterns when compared with *E. terrapinae*. Our study confirmed that the protein analysis is a valuable taxonomic criterion of *Entamoeba* species and showed that the disc electrophoresis of soluble amoebal proteins clearly differentiated *E. invadens* from *E. terrapinae* and may be used in epidemiological study.

The results of our experimental infection of grass snakes with amoebae isolated from a turtle *Pseudomys ornata callirostis* proved the amoebae to be pathogenic. This observation, as well as the results of amoebal protein analysis, showed that the source of epizootic fatal amoebiasis of snakes observed recurrently at the Poznań Zoological Gardens were infected turtles which had been exposed together with the snakes in the same vivaria.

REFERENCES

Davis B. J. and Ornstein L. 1959: A new high resolution electrophoresis method. Deliv. at Soc. for Study of Blood at N. Y. Acad. Med., Martch 24, 1959.

Donaldson M., Heyneman D., Dempster R. and Garcia L. 1975: Epizootic of fatal amebiasis among exhibited snakes: epidemiologic, pathologic, and chemotherapeutic considerations. Am. J. Vet. Res., 36, 807–817.

Golec W. and Kasprzak W. 1977: Ueber einige Erkrankungen bei Reptilien im Zoo Poznań. Verh. XIX Int. Symp. Erkr. Zootiere, 18–22 May 1977, Poznań, Poland, 65–67.

Hill W. C. and Neal R. A. 1954: An epizootic due to *Entamoeba invadens* at the Gardens of the Zoological Society of London. Proc. Zool. Soc. London, 123, 731–737.

Jacobson E., Clubb S. and Greiner E. 1983: Amebiasis in red-footed tortoises. J. Am. Vet. Med. Assoc., 183, 1192–1194.

Lowry O., Rosenbrough N. J., Farr A. L. and Randall R. J. 1951: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Meerovitch E. 1958: A new host of *Entamoeba invadens* Rodhain, 1934. Can. J. Zool., 36, 423–427.

Meerovitch E. 1961: Infectivity and pathogenicity of polyxenic and monoxenic *Entamoeba invadens* to snakes kept at normal and high temperatures and the natural history of reptile amoebiasis. J. Parasitol., 47, 791–794.

Mishra G. S. and Gonzales J. P. 1978: Les parasites des tortues d'eau douce en Tunisie. Archs Inst. Pasteur Tunis, 55, 303–326.

Pavlova E. A. 1938: (Sur les méthodes de la culture d'*Entamoeba histolytica* — An abstract in French). Med. Parazit. Parazit. Bol., 7, 111.

Said-Fernández S. and López-Revilla R. 1976: Electrophoretic characterization of proteins from four axenic *Entamoeba strains*. In: Amibiasis — Amoebiasis, (eds. B. Sepulveda and L. S. Diamond), Publ. Instituto Mexicano Seguro Social, Mexico, 1976, 104–111.

http://rcin.org.pl

Steck F. 1961: Amoebendysenterie bei Reptilien. Zbl. Bakteriol. Parasitol. Infektionskr. Hyg., Orig. A, 181, 551–553.
Whitney P. J., Vaughan J. G. and Heale J. B. 1968: A disc electrophoretic study of the proteins of *Verticillium albostratum*, *V. dahliae* and *Fusarium oxysporium* with reference to their taxonomy. J. Exp. Bot., 19, 415–426.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28. No. 3–4, pp. 293–302 (1989)

Trypanoplasma ompoki sp. n. from Freshwater Fishes in Kerala, India, with Observations on its Vector-Phase Development and Transmission

K. RAMANANDAN SHANAVAS, P. RAMACHNDRAN and K. P. JANARDANAN

Parasitology Laboratory, Department of Zoology, University of Calicut, Kerala-673 635, India

Received on 17th August, 1988, *revised on* 14th November, 1988

Synopsis. Trypanoplasma ompoki sp. n., recovered from the blood of *Ompok bimaculatus* (Bloch) and *Clarias dussumieri dussumieri* Valenciennes, two Indian freshwater fishes, is described in detail. There is evidence, that *Hemiclepsis marginata* (Mueller), a freshwater leech, is the natural vector of *T. ompoki*. Development is in the crop. Repeated multiplication of the trypanoplasms produces smaller forms, which transform themselves into metacyclics. The metacyclics invade the proboscis sheath and its vicinity. We succeeded in obtained experimental infection by exposing parasite-free *C. d. dussumieri* to experimentally infected *H. marginata*. Our attempts to transmit the infection to *Channa punctatus* (Bloch) and *Heteropneustes fossilis* (Bloch) under identical conditions were not successful *T. ompoki* has thus some degree of host specificity.

The information on trypanoplasms of Indian freshwater fishes is limited to the taxonomic reports on 3 species by Mandal (1979) and Joshi (1982). We came across a trypanoplasm in the blood of *Ompok bimaculatus* (Bloch) and *Clarias dussumieri dussumieri* Valenciennes, collected from Iritty in the Cannanore district of Kerala, during the course of a survey for piscine haemoprotozoans. This *Trypanoplasma*, on critical study, proved to be a new species; it is described in detail and reported here as *Trypanoplasma ompoki* sp.n. The present paper presents also our observations on its vector-phase development in *Hemiclepsis marginata* (Mueller), and the results of our attempts to transmit the flagellate from infected leeches to 3 fish species.

http://rcin.org.pl

Material and Methods

The fishes, *Ompok bimaculatus* and *Clarias dussumieri dussumieri*, were collected from different parts of the Cannanore and Malappuram districts of Kerala, from June to November 1985. The fishes were maintained in aquarium tanks, on a diet of beef liver and/or boiled egg-white. A drop of blood obtained by caudal artery puncture was mixed with 0.75% saline on a slide and examined to detect parasitemia. Blood from cardiac or caudal artery puncture was smeared on slides and stained with Giemsa to observe morphology of the flagellates.

Hemiclepsis marginata were collected from an irrigation canal in Iritty from June to August 1985. The leeches were found attached to fallen leaves and occasionally of fishes; many were brood leeches. The brood leeches were maintained individually in glass bowls with filtered pond water, at laboratory temperatures (27–29°C). Newly hatched leeches remained attached to the ventral surface of the mother. Eight to 12 days old leeches, ready to feed, were exposed to fishes. These leeches began to reproduce in the laboratory after 3 or 4 blood meals.

Newly emerged and 18–22 days old (fed once on an unifected fish and then starved) leeches, taken from the laboratory cultures, were allowed to feed on an infected *O. bimaculatus* or *C. d. dussumieri*. The engorged leeches were kept at laboratory temperatures (27–29°C), in glass bolws with filtered pond water, dissected at intervals and examined for the developmental forms of the trypanoplasm. Leeches pressed under coverslips and examined under 10× objective of a microscope usually revealed the sites of infection. The proboscis along with its sheath was microscopically dissected and examined for flagellates invading the proboscis sheath. The crop was punctured, crop contents were mixed with a minimum quantity of 0.75% saline, and examined under phase-contrast optics. Giemsa stained smears of the proboscis sheath and crop contents were also prepared and studied.

Experimental transmission of the flagellate from the leech to parasite-free *C. d. dussumieri*, *Channa punctatus* and *Heteropneustes fossilis* was attempted. The host fishes, maintained in the laboratory for more than a month and proved uninfected by periodic blood examinations were exposed to infected leeches. Blood of the exposed fishes, was examined at intervals for the *Trypanoplasma*.

Sketches were made with the aid of a camera lucida. Measurements were taken from camera lucida sketches, following the method outlined by Bower and Woo (1977). Ten measurements were taken from a specimen: distance from anterior to posterior end of body, excluding free flagella (PA), body width at the level of nucleus (W), length of anterior free flagellum (AF), length of posterior free flagellum (PF), perpendicular measurements through nucleus (NM), distance between anterior edge of nucleus and anterior tip of body (NA), length of kinetoplast (LK), width of kinetoplast (WK) and distance between anterior edge of kinetoplast and anterior tip of body (KA). The body ratio W/PA was also calculated. The descriptions are based on measurements of a minimum of 20 specimens.

Results

Trypanoplasma ompoki sp. n.

Eight of 20 *Ompok bimaculatus*, collected from irrigation canals and rivulets in Iritty in the Cannanore district of Kerala, were positive for

http://rcin.org.pl

the trypanoplasm. Blood of 7 out of 22 *Clarias dussumieri dussumieri*, from the same localities, was also positive for the infection.

Description

Trypanoplasm (Fig. 1. *1–3*; Pl. I 1–5) with elongated body, broad at anterior half, tapering gradually toward the posterior region; anterior end broadly round, posterior end narrow and blunt. A narrow undulating membrane usually discernible. Anterior flagellum long, measuring 19.5–23.5 μm; posterior flagellum (recurrent flagellum) borders the undulating membrane and emerges from the posterior end as a moderately long free flagellum, 13.5–18 μm long; flagella stain reddish-violet with Giemsa. Cytoplasm coarsely granular, nonhomogeneous, basophilic, stains blue; dark-purple staining, large, chromophilic granules present in cytoplasm; the granules remain scattered in the cytoplasm, occasionally in aggregarions of 2–4. Nucleus 3–4.5 μm from anterior end, elongated, granular stains reddish-purple. Kinetoplast 1.5–3 μm from anterior end, oblong, roughly rectangular or comma-shaped, attached to the body margin opposite to the nucleus and stains dark-blue.

Measurements (in micrometers; range in parentheses):
PA: 25.65 (21–30); W: 6.86 (5.25–8); AF: 21.53 (19.5–23.5); PF: 15.23 (13.5-18); NM: 9.28 × 2.36 (8.25-11.5 × 2-3); NA; 3.81 (3-4.5); LK: 5.63 (5–6.75); WK: 1.55 (1–2.25); KA: 1.95 (1.5–3); W/PA: 0.27 (0.21–0.33).

Type host:	*Ompok bimaculatus* (Bloch)
Additional host:	*Clarias dussumieri dussumieri* Valenciennes
Type locality:	Iritty, Cannanore district, Kerala, India
Invertebrate vector:	*Hemiclepsis marginata* (Mueller)
Date of collection:	June to November 1985
Holotype:	Deposited in the parasitological collection in the Department of Zoology, University of Calicut, Kerala, India

Development of *Trypanoplasma ompoki* in the leech, *Hemiclepsis marginata*

Developmental forms of *T. ompoki* were observed in *H. marginata*, fed on an infected *O. bimaculatus* or *C. d. dussumieri* and dissected at different intervals. The crop contents of the leeches, dissected immediately after engorgement, contained active flagellates. The flagellates, in Giemsa-stained preparations, resembled the bloodstream forms, but were

http://rcin.org.pl

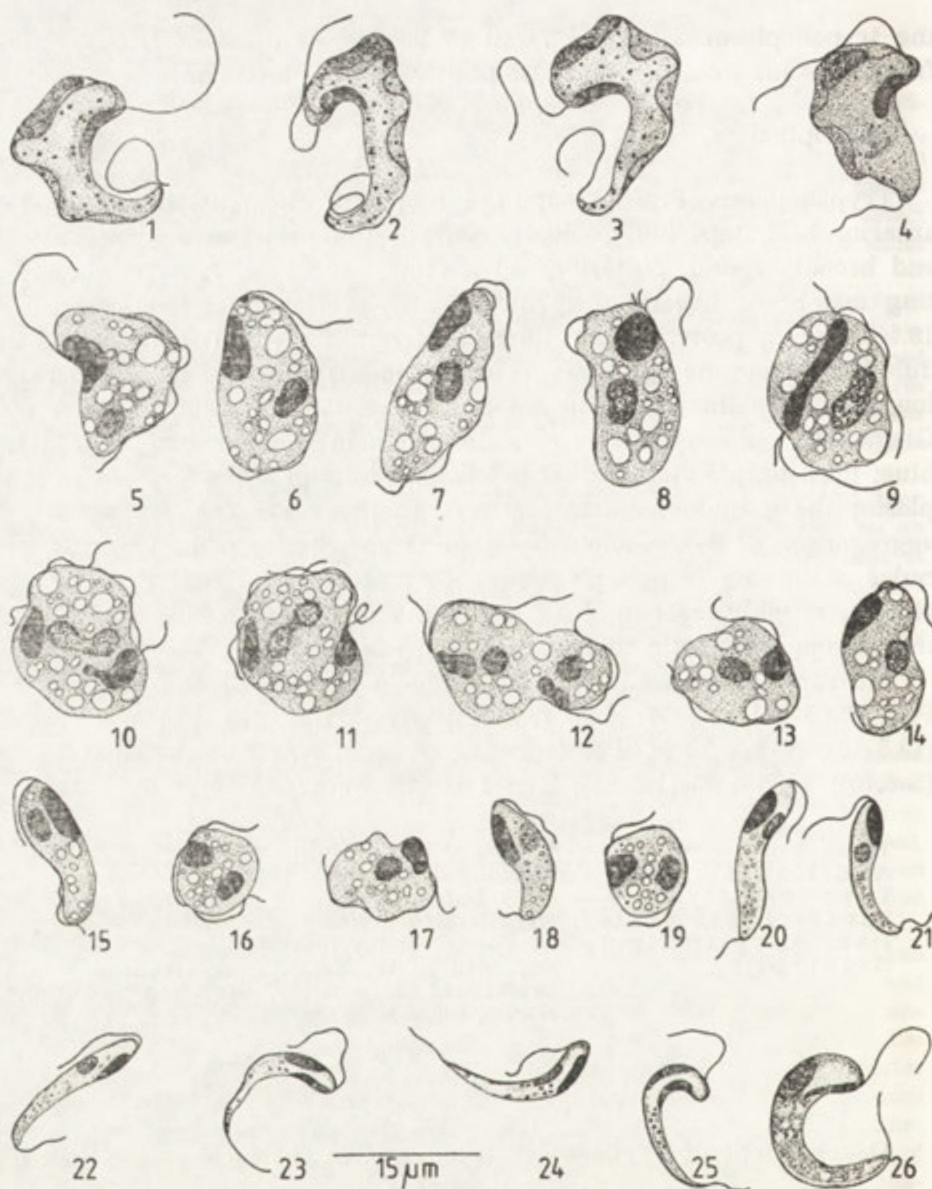


Fig. 1. *1–26 Trypanoplasma ompoki* sp. n. *1, 2* — Blood forms from *Ompok bimaculatus*, *3* — Blood form from *Clarias dussumieri dussumieri*, *4–23* — Developmental forms from crop conents of *Hemiclepsis marginata* at different intervals after blood meal, *4* — Immediately after the blood meal, *5–7* — 2 h post-feeding, *8–12* — 4 h post-feeding, dividing flagellates, *13, 14* — 10 h post-feeding, *15–17* — 20 h post-feeding, *15* — Elongated form, *16* — Roughly round form, *17* — Irregular form, *18, 19* — 33 h post-feeding, *20* — 72 h post-feeding, slender flagellate, *21 22* — 84 h post-feeding, slender flagellates, *23* — 97 h post-feeding, metacyclic flagellate, *24, 25* — 115 h post-feeding, metacyclics from proboscis sheath, *26* — Blood form in the heart blood of *C. d. dussumieri*, 36 h after infection

http://rcin.org.pl

shorter and wider (PA: 15.75–18.75 μm; W: 9–12 μm) (Fig. 1 *4*). The chromophilic granules were reduced in number and size.

Leeches dissected at 2 h post-feeding (p. f.) contained active flagellates, which were continuously changing their shape. The flagellates showed no directional motion. The anterior flagellum exhibited a continuous lashing activity. The posterior flagellum, bordering the body margin, had an actively lashing short free end. The flagellates in Giemsa--stained preparations were irregular, oval or oblong in shape, measuring 15–25 μm × 6.75–13.5 μm (Fig. 1 *5–7*). The cytoplasm was highly basophilic, stained deep-blue and contained several scattered, round to oval, unstained areas or vacuoles. The nucleus was roughly round, granular and stained purple. The kinetoplast was oval or irregular in shape and stained dark-blue.

At 4 h p. f., the crop contents contained dividing flagellates in various stages of binary fission (Fig. 1 *8–12*). Two new flagella grew out from the kinetoplast, and continued to elongate. The kinetoplast elongated next and was the first to divide in fission; the nucleus now contained short, thick chromatin strands. Nuclear division followed the dividion of the kinetoplast. Cytoplasmic division produced 2 individuals of equal or unequal size.

At 10 h p. f., the crop contained flagellates similar to those observed at 2 h p. f. and smaller forms (Fig. 1 *13* and *14*) measuring 12–16.5 μm × 7.5–10.5 μm. The cytoplasm of the smaller forms was less basophilic and contained a lesser number of vacuoles. Dividing forms were common.

At 20 h p. f., a large number of pyriform or oblong flagellates, active in wet preparations, exhibiting pulsation of body and slow progressive motion, were observed. The anterior flagellum lashed continuously; the short free portion of the posterior flagellum was usually visible. Three types of flagellates were seen in Giemsa-stained preparations: (1) elongated, slightly curved forms (Fig. 1 *15*) with blunt ends, measuring 10.5–16.5 μm × 4.5–6 μm, (2) roughly round forms (Fig. 1 *16*), measuring 7.5–9.75 μm × 6–9 μm and (3) irregular-shaped forms (Fig. 1 *17*), measuring 10.5–12 μm × 6–10.5 μm. The cytoplasm of these forms was coarsely granular, contained several vacuoles and stained blue. The nucleus was roughly oval and stained light-purple. The kinetoplast was roughly oval or elongated and stained dark-blue.

At 33 h p. f., a large number of undivided and dividing flagellates was observed. The flagellates in fresh preparations were long, slightly curved forms with bluntly round ends, and showed progressive motion by whipping of the anterior flagellum. The short free portion of the posterior flagellum exhibited slow lashing activity or was held rigid at an angle to the body. In Giemsa-stained preparations, elongated, slighly

http://rcin.org.pl

curved forms, with rounded ends and roughly rounded forms were observed. The elongated forms (Fig. 1 *18*) measured 9–18 μm × 3–3.75 μm and the roughly rounded forms (Fig. 1 *19*) measured 7.5–12 μm × 6–9 μm. The cytoplasm of these forms contained a few, scattered, dark-purple staining granules and small vacuoles.

At 72 h p. f., the flagellates in the crop of the leeches increased in number. The flagellates in fresh preparations were morphologically similar to those observed at 33 h p. f., but were slightly longer. These forms exhibited rapid progressive motion. The flagellates in Giemsa-stained preparations (Fig. 1 *20*) were slender, elongated and slightly curved with blunt ends; the posterior end was narrow. Roughly rounded forms were also found; dividing forms were common. The cytoplasm of the flagellates now stained light-blue and contained several relatively large, scattered, dark-purple-staining granules and minute vacuoles.

At 84 h p. f., the crop contents contained enormous number of flagellates resembling those observed at 72 h p. f.; in addition more slender forms (Fig. 1 *21 22*), with narrow posterior ends, longer flagella and finely granular cytoplasm were also observed. Dividing forms were absent.

Metacyclic forms appeared in the crop contents at 97 h post-feeding. The metacyclic forms (Fig. 1 *23*; Pl. I 6,7) were slender, elongated, with rounded anterior and sharply pointed posterior ends and measured 16.5–20.5 μm × 1.5–2.25 μm. The anterior flagellum measured 9–13.5 μm. The posterior flagellum bordered the body and emerged at the posterior end as a short free flagellum, measuring 3–6 μm. The cytoplasm was finely granular, devoid of vacuoles, contained a few deep-purple-staining granules and stained pale-blue. The nucleus was roughly oval or elongated, 2.25–6 μm from the anterior end, and stained light-pink. The kinetoplast was elongated, located close to the anterior end, attached to the body margin opposite the nucleus and stained dark-blue.

At 115 h p. f., the proboscis sheath of the leeches contained a few metacyclics (Fig. 1 *24, 25*). The crop and its caeca contained swarms of metacyclics and a few forms resembling those observed at 84 h post-feeding. The forms in the proboscis sheath continued to increase in number, and by the 7th day p. f., the proboscis sheath was swollen and filled with the metacyclics. Metacyclics could be observed throughout the crop and its caeca for up to 28 h after the complete disappearance of the blood meal; thereafter the flagellates occurred only in the proboscis sheath and its vicinity. The metacyclics remained in the proboscis sheath for at least 27 days after the infected feed.

Infected leeches retained the infection even after 2 successive uninfective blood meals. Metacyclics were not observed in the proboscis

http://rcin.org.pl

sheaths of the leeches, dissected immediately after the uninfected feed; they were in the crop contents. Metacyclics reappeared in the proboscis sheath in 48–72 h post-feeding.

Experimental Transmission

Experimental transmission of *T. ompoki* to parasite-free *C. d. dussumieri* was accomplished by exposing the fishes to the leeches with metacyclic forms. Three *C. d. dussumieri* were exposed to leeches fed 6–9 days previously on an infected *O. bimaculatus* and another *C. d. dussumieri* was exposed to leeches fed 12 days previously on an infected *C. d. dussumieri*. Ten leeches fed on each fish. Flagellates were observed in the heart blood of a *C. d. dussumieri*, dissected 36 h later. Peripheral blood infection was detected in 2 *C. d. dussumieri* on the 6th day and in the remaining one on the 7th day.

The flagellates observed in the heart blood of *C. d. dussumieri* were elongated, slender forms with broadly rounded anterior and narrow posterior ends and measured 21–25.5 μm × 3.75–4.5 μm (Fig. 1 *26*; Pl. I 8,9). The anterior flagellum measured 12–15 μm, and the free portion of the posterior flagellum measured 4.5–6.75 μm. The cytoplasm was granular, with a few vacuoles and stained light-blue. The nucleus was oval, 4.5–6 μm from the anterior end, and stained rose-red. The kinetoplast was elongated and bar-like, located close to the anterior end, and stained dark-blue. The peripheral blood forms first observed in other experimentally infected fishes were broader with longer free flagella. Flagellates, morphologically similar to the typical blood forms, were observed on the 3rd day after the manifestation of blood parasitemia.

Attempts were made to transmit the infection to 2 uninfected *Channa punctatus* and 3 *Heteropneustes fossilis*, by exposing the fishes to the leaches with metacyclic forms. Ten leeches fed on each fish. Flagellates were not detected in any of the fishes though periodic blood examinations were made for up to 30 days following feedings by the infected leeches.

Discussion

The family *Cryptobiidae* Poche includes 2 genera, *Cryptobia* and *Trypanoplasma*. Leidy (1846) erected *Cryptobia* for the biflagellate kinetoplastids of snails. The genus also now includes the cryptobiids from the gills and skin of freshwater fishes, the digestive system of marine fihes and internal organs of various invertebrates (Lee et al. 1985). Laveran and Mesnil (1901), however, established *Trypanoplasma* for biflagellate bloodstream kinetoplastids of fishes. The 2 genera are morphologically similar, but biologically different. *Cryptobia* are trans-

http://rcin.org.pl

mitted directly, while *Trypanoplasma* are transmitted by leech vectors. On the basis of morphological similarity, Crawley (1909) considered *Trypanoplasma* as a synonym of *Cryptobia*, but based on biological difference Laveran and Mesnil (1912) retained *Trypanoplasma*. However, researchers remained divided in their opinion and continued to assing biflagellate blood-stream kinetoplastids to both *Cryptobia* and *Trypanoplasma*. Becker (1977) in his review on flagellate parasites of fishes accepted *Trypanoplasma* for these cryptobiids parasitizing the blood of fishes and transmitted by leeches and *Cryptobia* for all others associated with fishes. Burreson (1979) also expressed the same view. We accept this system and assign the present biflagellate haemoprotozoan of fishes to *Trypanoplasma*.

Becker (1977) listed 21 species of *Trypanoplasma* from the freshwater fishes of the northern hemisphere. The present species requires comparison with *T. clariae*, the only species reported by Mathis and Leger (1910) from a clariid fish, *Clarias macrocephalus*. A comparison of the dimensions (Table 1) of the 2 species shows that the species under study is significantly different from *T. clariae*.

Table 1

Comparison of dimensions of trypanoplasms

Parasite	*Cryptobis indica* Mandal, 1979	*Trypanoplasma clariae* Mathis and Leger, 1910	*T. ompoki* sp. n.
Host	*Mystus vittatus*	*Clarias macrocephalus*	*Ompok bimaculatus* and *Clarias dussumieri dussumieri*
PA	28.5 (25–30)	32 (29–35)	25.65 (21–30)
W	8.5 (6–10.5)	10.5 (9–12)	6.86 (5.25–8)
AF	25 (21–30)	22 —	21.53 (19.5–23.5)
PF	10.5 (9–12)	10 —	15.23 (13.5–18)
NM	7.5×3 (5–8.5×2.5–3.5)	— (5–7×2)	9.28×2.36 (8.25–11.5×2–3)
LK	5.5 (5–6)	4.5 —	5.63 (5–6.75)
WK	1.5 (0.75–2)	1.5 —	1.55 (1–2.25)

Measurements in micrometer; range in parentheses. PA — distance from anterior to posterior end of body, excluding free flagella; W — body width at the level of nucleus; AF — length of anterior free flagellum; PF — length of posterior free flagellum; NM — perpendicular measurements through nucleus; LK — length of kinetoplast; WK — width of kinetoplast

http://rcin.org.pl

Three species of trypanoplasms have previously been reported from Indian freshwater fishes. Mandal (1979) described *Cryptobia indica* from the blood of *Mystus vittatus* in Bengal. Joshi (1982) described *Trypanoplasma aori* from *Mystus aor* and *T. atti* from *Wallago attu* in Lucknow. The present species resembles most *C. indica* in general body form, but differs in dimensions (Table 1) and cytomorphological features. The cytoplasm of the species under study is coarsely granular and contains large, prominent, scattered, chromophilic granules, whereas that of *C. indica* is finely granular, without any chromophilic granules. In addition, the species from *O. bimaculatus* and *C. d. dussumieri* differs from all other *Trypanoplasma* from freshwater fishes, and is, therefore, reported here as new species and named *Trypanoplasma ompoki* sp. n.

Vector-phase development of *T. ompoki* has been observed in the crop of *Hemiclepsis marginata*. The flagellate begins to divide in the crop a few hours after ingestion. Multiplication produces smaller forms, which transform themselves into slender metacyclics. The metacyclics migrate into the proboscis sheath and its vicinity, where they occur after the complete digestive removal of the bloodmeal. The vector-phase development of *T. ompoki* is similar to those of other marine (Burreson, 1979, 1982) and freshwater (Lom, 1979) trypanoplasms.

Developmental stages of *T. ompoki* have been observed in several wild-caught *H. marginata*. On several occasions we recovered the leeches from infected fishes. Furthermore, *T. ompoki* has been successfully transmitted from experimentally infected *H. marginata* to *C. d. dussumieri*. We, therefore, suggest *H. marginata* as a natural vector of *T. ompoki*. This forms the first report on the development and transmission of a trypanoplasm from Indian freshwater fishes.

Infection with *T. ompoki* could not be observed in *Channa punctatus* and *Heteropneustes fossilis* collected from the same aquatic habitats where infected *O. bimaculatus* and *C. d. dussumieri* and *H. marginata* were collected. Attempts to transmit the trypanoplasm to *C. punctatus* and *H. fossilis* by exposing them to experimentally infected leeches also were not successful. Therefore, *T. ompoki* is possibly a species with some degree of host specificity. Our observation is in accord with that of Bower and Woo (1977) who found *Cryptobia catostomi* of *Catostomus commersoni* refractive to 16 species of fishes.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. Jacqueline Madill, National Museum of Natural Sciences, Canada, for taxonomic identification of the leech vector. One of the authors (K.R.S.) is grateful to the authorities of the University of Calicut for financial support.

http://rcin.org.pl

REFERENCES

Becker C. D. 1977: Flagellate parasites of fish. In: Parasitic Protozoa, I, (ed. J. P. Kreir), Academic Press Inc., New York, 357–416.

Bower S. M. and Woo P. T. K. 1977: Morphology and host specificity of *Cryptobia catostomi* n. sp. (*Protozoa* : *Kinetoplastida*) from white sucker (*Catostomus cammersoni*) in Southern Ontaria. Can. J. Zool., 55, 1082–1092.

Burreson E. M. 1979: Structure and life cycle of *Trypanoplasma beckeri* sp. n. (*Kinetoplastida*), a parasite of the cabezon, *Scorpaenichthys marmoratus*, in Oregon coastal waters. J. Protozool., 26, 343–347.

Burreson E. M. 1982: The life cycle of *Trypanoplasma bullocki* (*Zoomastigophora: Kinetoplastida*). J. Protozool., 29, 72–77.

Crawley H. 1909: Studies on blood and blood-parasites. II. The priority of *Cryptobia* Leidy, 1846 over *Trypanoplasma* Laveran and Mesnil, 1901. Bull. Bur. Animal Ind., 119, 16–20.

Joshi B. D. 1982: Three new species of haematozoans from freshwater teleosts (Pisces). Proc. Indian Acad. Sci. (Anim. Sci.), 91, 397–406.

Leveran A. and Mesnil F. 1901: Sur les flagellés à membrane ondulante des poissons (Genres *Trypanosoma* Gruby et *Trypanoplasma* n. gen.). C. R. Acad. Sci., Paris, 133, 670–675.

Laveran A. and Mesnil F. 1912: Trypanosomes et Trypanosomiases. Second edition. Masson et Cie, Paris.

Lee J. J., Hunter S. M. and Bovee E. C. (eds.) 1985: An Illustrated Guide to the *Protozoa*. Society of Protozoologists, Lawrence, Kansas.

Leidy J. 1846: Description of a new genus and species of entozoa. Proc. Acad. Nat. Sci., Philad., 3, 100–101.

Lom, 1979: Biology of trypanosomes and trypanoplasms of fish. In: Biology of Kinetoplastida, 2, (eds. W. H. R. Lumsden and D. A. Evans), Academic Press Inc., New York, 269–337.

Mandal A. K. 1979: Studies on the haematozoa of some catfishes belonging to the genus *Mystus* (Scopoli) from India. Bull. Zool. Surv. India, 2, 17–23.

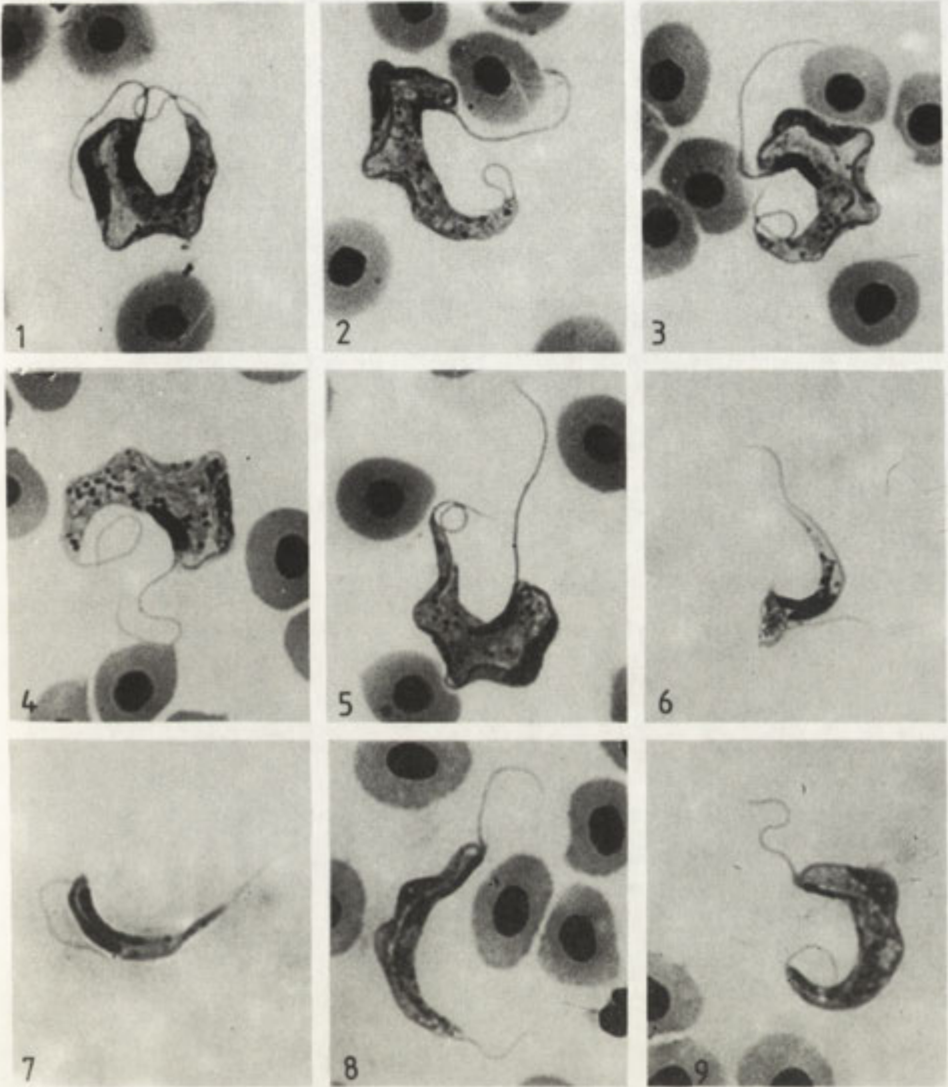
Mathis C. and Leger M. 1910: *Trypanoplasma* d'un poisson du Tonkin, *Clarias macrocephalus*. C. R. Soc. Biol., 69, 351–353.

EXPLANATION OF PLATE I

1–9 *Trypanoplasma ompoki* sp. n.

1,2: Blood forms from *Ompok bimaculatus*
3–5: Blood forms from *Clarias dussumieri dussumieri*
6,7: Metacyclics from crop contents of *Hemiclepsis marginata*, 97 h after blood meal
8,9: Blood forms in the heart blood of a *C. d. dussumieri*, 36 h after infection

http://rcin.org.pl



K. Ramanandan Shanavas et al. auctores phot.

http://rcin.org.pl

http://rcin.org.pl

ACTA PROTOZOOLOGICA
Vol. 28, No. 3-4, pp. 303--310 (1989)

Observations on *Stephanospora paratelphusae* gen. n., sp. n. (*Apicomplexa*: *Cephalina*) from the Freshwater Crab, *Paratelphusa hydrodromous* (Herbst)

S. PREMA and K. P. JANARDANAN

Parasitology Laboratory, Department of Zoology, Calicut University, 673 635 Kerala, India

Received on 15th June, 1988, *revised on* 14th October, 1988

Synopsis. Stephanospora paratelphusae gen. n., sp. n. from the intestine of the freshwater crab, *Paratelphusa hydrodromous* (Herbst), collected from Kerala, India is described in detail and its systematic position discussed. The gregarine has biassociative and occasionally triassociative sporadins, and ring- or crown-like epimerite with closely packed longitudinal striations. The epimerite is retained throughout the vegetative stages, and its structure is unique among the known cephaline gregarines. Trophic stages of the gregarine have been observed and reported. Gametocysts were recovered from the posterior part of the midgut. The cysts develop and release mulberry-like gymnospores into the gut lumen by simple rupture. Further development of the gymnospores was not observed.

The gregarines known from crabs of the world are placed under 4 genera, *Porospora* Schneider, 1875, *Nematopsis* Schneider, 1892, *Cephaloidophora* Mavrodiadi, 1908 and *Pachyporospora* Théodoridès, 1961. More than 42 species belonging to these genera have been reported from crabs. The contributions came from Diesing (1851), Schneider (1875, 1892), Frenzel (1885), Léger and Duboscq (1907, 1911a, b), Beauchamp (1910), Watson (1916), Hatt (1931), Pearse (1932, 1933), Prytherch (1938), Ball (1938, 1948, 1951, 1959, 1963), Cuckler and Fichter (1939), Sprague (1949, 1954), Bogolepova (1953), Tuzet and Ormières (1961), Théodoridès (1961, 1962, 1967, 1979), Ormières (1968), Vivárès and Rubió (1969), Vivárès (1970, 1971, 1978) and Desportes et al. (1977).

While studying the gregarines of crustaceans we came across a spe-

http://rcin.org.pl

cies of cephaline gregarine from the intestine of a freshwater crab, *Paratelphusa hydrodromous* (Herbst). The structure and biology of the gregarine showed that it could not be included within any of the 4 genera reported from crabs. The gregarine is described here as *Stephanospora paratelophusae* gen. n., sp. n. for reasons discussed elsewhere.

Material and Methods

The freshwater crabs, *Paratelphusa hydrodromous*, were collected from the paddy fields at Idimuzhikal and Edappal in the Malappuram district of Kerala. The specimens were immediately examined for their gregarines, or maintained alive and studied at convenience. The trophozoites and sporadins were studied from smears of infected midguts, fixed in Schaudinn's fluid and stained with Heidenhain's hematoxylin. Pieces of the midgut, fixed in Bouin's fluid and sectioned at 5 μm were stained as above for studying the intracellular stages of development. Gametocysts were collected from the posterior part of the midgut and maintained in moist chamber for further development. Various development stages of live gregarines were studied by staining them supravitally with neutral red or methylene blue.

Sketches were made with the aid of a camera lucida; descriptons are based on the measurements of a minimum of 20 specimens.

Results

Stephanospora paratelphusae gen. n., sp. n.

Description

Sporadins (Fig. 1 *1*; Pl. I 1): Biassociative, linear, opaque, dull-white; syzygy early; occasionally three sporadins in a linear chain (Pl. I 2). Epimerite ring- or crown-like, retained throughout association. The length of association ranges from 504 to 1120 μm (640.6 μm).

Primites: Stout, narrow anteriorly, broader posteriorly; epimerite ring- or crown-like, with closely packed longitudinal striations (Pl. I 3); epimerite 15.4 μm thick; protomerite hemispherical, with the width extending the length; protomerite epicyte uniformly thick, longitudinally striated, striations continuous with those on deutomerite; constriction at septum inconspicuous. The deutomerite broadens gradually from the septum and ends in a flat caudal end; epicyte hyaline, uniformly thick; endoplasm granular. The nucleus spherical, feebly visible in fresh sporonts, variable in position; endosome round, made of numerous granules; two extra-endosomal basophilic bodies present. The nucleus in a 320 μm long primite measured 48 μm in diameter.

http://rcin.org.pl

S a t e l l i t e s: Stout, broader at the anterior region, gradully tapering to a broadly round caudal end. Protomerite not discernible in mature satellites, rectangular, if present; epicyte hyaline, uniformly thick, longitudinally striated; endoplasm granular. The nucleus spherical, feebly visible in fresh sporonts, variable in position; endosome round, made of numerous granules; two extra-endosomal basophilic bodies present. The nucleus in a 448 μm long satellite measured 48 μm in diameter.

M e a s u r e m e n t s (in micrometers): The measurements of sporadins (with mean in parentheses) are noted below:

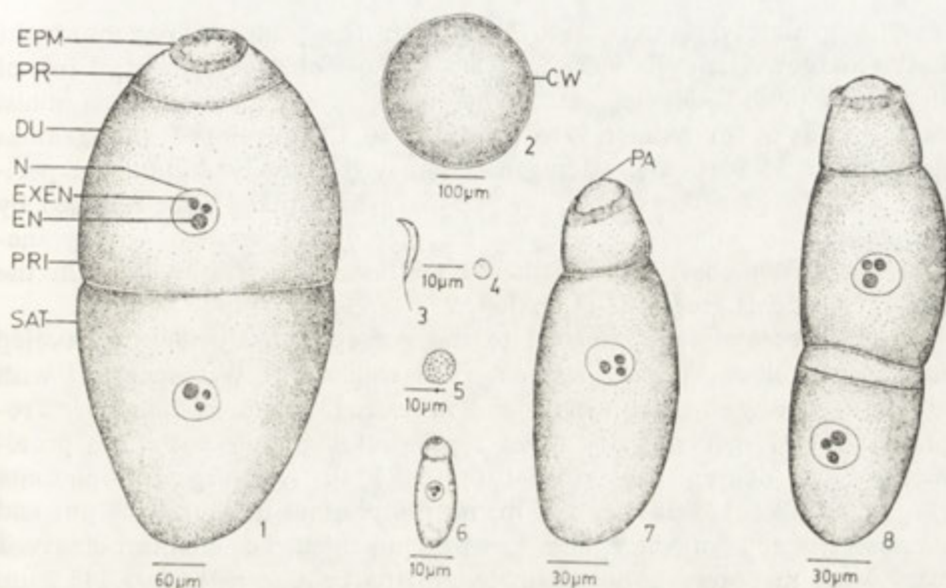


Fig. 1. *Stephanospora paratelphusae* gen. n., sp. n. 1 — Sporadins, 2 — Gametocyst, 3 — Microgamete, 4 — Macrogamete, 5 — Gymnospore, 6–7 — Trophoites, 8 — Small association
CW — cyst wall, DU — deutomerite, EN — endosome, EPM — epimerite, EXEN — extra-endosomal body, N — nucleus, PA — protomerite apex projecting through the epimerite, PR — protomerite, PRI — primate, SAT — satellite

P r i m i t e s: Total length (TL), 254.1–624 (338); protomerite length (PL), 38–112 (66); protomerite width (PW), 100.1–218.4 (158.9); deutomerite width (DW), 177.1–331.1 (267).

S a t e l l i t e s: Total length (TL), 231–496 (320); deutomerite width (DW), 176–323.4 (245.3).

R a t i o s (of primites): PL : TL = 1 : 5.4; PW : WD = 1 : 1.7.

G a m e t o c y s t s (Fig. 1 2): Gametocysts spherical or ovoid, opaque, milky-white; cyst wall thin. Line of association clear in early gametocysts.

http://rcin.org.pl

Measurements: Fresh gametocysts measured 352 by 416 μm — 512 by 448 μm (426.4 by 452 μm).

Gametes: Anisogamous; microgametes motile, with spindle-shaped body and a slender, drawn out tail (Fig. 1 *3*); macrogametes spherical, non-motile (Fig. 1 *4*).

Gymnospores (Fig. 1 *5*): Gymnospores spherical; uninucleated, spherical bodies arranged in a mulberry-like manner around a central, hyaline cytoplasm. Gymnospores measured 6 μm in diameter.

Life-cycle Stages

The gametocysts were recovered from the posterior region of the host's midgut. The cysts were in various stages of development; a few of them completed development. The gametocysts maintained in moist chamber, at room temperature (28 C to 30 C), developed anisogamous gametes in 24–30 hours. Microgametes moved actively around the periphery of the gametocyst. In 58–72 h, the gymnospores were released by simple rupture of the gametocysts. Further development of the gymnospores was not observed. Whether a molluscan host is involved in the life-cycle of this gregarine is unknown.

The sporozoites get attached to the gut epithelial cells and develop into trophozoites. The smallest observed trophozoite was spherical with a ring- or crown-like epimerite and measured 24 μm in diameter. Trophozoites of larger size developed septa dividing their body into protomerites and deutomerites (Fig. 1 *6*; PL. I 4). The largest trophozoite (Fig. 1 7) measured 184 by 24 μm; its protomerite measured 24 μm and deutomerite 152 μm; the epimerite was 8 μm thick. The smallest observed association measured 237.6 μm in length; its primite measured 145.2 μm and satellite 92.4 μm. The ring- or crown-like epimerite was 8 μm thick. The apex of the primite protomerite projected through the centre of the epimerite in the form of a knob (Fig. 1 *8*; PL. I 5,6). The deutomerite was rather cylindrical, measured 95.7 μm. The satellite was conical with a rectangular, 13.2 μm long protomerite and 79.2 μm long deutomerite. The nuclei were spherical, with round endosome and two extra-endosomal bodies. With further growth and differentiation the associating sporadins get transformed into gametocysts. Occasionally linear associations of 3 sporadins were observed in the intestinal lumen.

Toxonomic Summary

Diagnosis: Sporadins biassociative, occasionally triasociative, rather stout; epimerite ring-or crown-like, with closely packed longitudinal striations; epimerite present in trophozoites and primites of syzygies. Maximum length of association 1120 μm. Primite protomerite hemisphe-

http://rcin.org.pl

rical, with the width extending the length; deutomerite broadens behind the septum and ends in a flat caudal end. Satellite broader at the anterior, gradually tapering to a round caudal end. Nuclei spherical, with one endosome made of numerous granules; two extra-endosomal bodies present; gametocyst spherical or ovoid, with thin cyst wall; gymnospores spherical, with uninucleated, spherical bodies arranged in a mulberry--like manner around a central, hyaline cytoplasm.

Host: *Paratelphusa hydrodromous* (Herbst)
Location in host: Intestine
Type locality: Idimuzhikkal and Edappal, Malappuram district, Kerala (India)
Date of collection: June to October (1985 to 1987)
Holotype: To be deposited in the parasite collections, Parasitology Laboratory, Department of Zoology, University of Calicut, Kerala (India)

Discussion

The family Porosporidae Labbé, 1899 is unique among gregarines in that its members are hetoroxenous, with vegetative development in the digestive tract of a crustacean and sporogony in the connective tissues of a mollusc. The gametocysts release gymnospores, which perhaps develop into naked zoites or monozoic, resistant sporocysts in molluscs. The epimerite, if present, is spherical. The family includes the 3 genera, *Porospora* Schneider, 1875, *Nematopsis* Schneider, 1892 and *Pachyporospora* Théodoridès, 1961.

The known species of *Porospora* have relatively longer syzygies and the individuals have a strong tendency to remain isolated. In members of *Nematopsis*, the syzygy is precocious, typically with several individuals in strainght or forked chains. In older associations the primite and satellite may be enclosed in a common epicyte, while the satellite or satellites become a single, often multinucleated compartment. The genus *Pachyporospora* is distinguished in that the individuals in association become fused into a single multinucleated compartment. The epicyte has conspicuous folds also.

The present gregarine from the freshwater crab, *Paratelphusa hydrodromous*, has short, stout, biassociative sporadins, ring- or crown-like epimerite with closely packed longitudinal striations and mulberry-like gymnospores. The epimerite is retained throughout the vegetative stages and its structure is unique among the known cephaline gregarines. The gregarine is thus distinct from the members of the three genera under the family *Porosporidae*. Besides, this is the first world record of a ce-

http://rcin.org.pl

phaline gregarine from a freshwater crab. The gregarine is, therefore, placed in a new genus, *Stephanospora* [1], in the family *Porosporidae*, and reported as *Stephanospora paratelphusae* gen. n., sp. n., after its host.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. J. Théodoridès, Research Professor, Laboratoire d'Evolution, University of Paris, France, for suggesting the generic name and also for the most helpful suggestions in the preparation of the manuscript. Sincere thanks are noted here to the University Grants Commission, New Delhi, for financial assistance (No. F. 3–79/86 SR II).

REFERENCES

Ball G. H. 1938: The life history of *Carcinocetes hesperus* n. gen., n. sp., a gregarine parasite of the striped shore crab, *Pachygrapsus crassipes*, with observations on related forms. Arch Protistenkd., 90, 299–319.

Ball G. H. 1948: A gregarine from the sand-crab, *Emerita analoga*. J. Parasit., 34, 17 (Abstr. 23).

Ball G. H. 1951: The occurrence of a porosporid gregarine, *Nematopsis* sp. in the striped shore-crab, *Pachygrapsus crassipes*. Proc. Am. Soc. Protozool., 2, 18 (Abstr. 47).

Ball G. H. 1959: Some gregarines from crustaceans taken near Bombay, India. J. Protozool., 6, 8–13.

Ball G. H. 1963: *Cephaloidophora carpilodei* n. sp. and *C. pinguis* n. sp., gregarines of a xanthid crab in Hawaii. J. Protozool., 10, 321–327.

Beauchamp P. de 1910: Sur une grégarine nouvelle du genre *Porospora*. C. R. Acad. Sci., 151, 997–999.

Bogolepova I. I. 1953: Gregarines from the Gulf of Peter the Great. Trudy Zool. Inst. Acad. Sci. USSR., 13, 38–56.

Cuckler A. C. and Fichter E. 1939: Gregarines from the crab, *Cancer productus*. J. Parasit., 25, 184.

Desportes I., Vivarès C. P. and Théodoridès J. 1977: Intérêst taxonomique de l'ultrastructure épicytaire chez *Ganymedes* Huxley, *Porospora* Schneider et *Thiriotia* n. g., Eugrégarines parasites de Crustacés. Ann. Sci. Nat. Zool., 12e serie, 19, 261–277.

Diesing C. M. 1851: Systema helminthum, 2. Vindobonae, Berlin.

Frenzel J. 1885: Über einige in Seethieren lebende Gregarinen. Arch Mikrosk. Anat. Entw. Mech., 24, 545–588.

Hatt P. 1931: L'évolution des porosporides chez les mollusques. Arch Zool. Exp. Gén., 72, 341–415.

Léger L. and Duboscq O. 1907: L'évolution des *Frenzelina* (N. G.), gregarines intestinales des crustacés décapodes. C. R. Acad. Sci., 145, 773–774.

Léger L. and Duboscq O. 1911 a: Deux grégarines de crustacés. *Porospora portunidarum* Frenz. et *Cephaloidophora maculata* n. sp. Archs Zool. Exp. Gén., 6, 59–60.

Léger L. and Duboscq O. 1911 b: Deux nouvelles espèces de grégarines appartenant au genre *Porospora*. Ann. Univ. Grenoble, 23, 401–404.

Ormières R. 1968: *Pachyporospora retorta* n. sp. grégarina parasite de *Macropipus holsatus* F. crustacé-décapode. Ann. Parasit. Hum. Com., 43, 413–419.

Pearse A. S. 1932: Observations on the ecology of certain fishes and crustaceans along the bank of the Matla River at Port Canning. Rec. Indian Mus., 34, 289–298.

Pearse A. S. 1933: Parasites of Siamese fishes and crustaceans. I. Siam. Soc., 9, 179–191.

[1] From the greek: στεφυη[α],η = Crown

http://rcin.org.pl

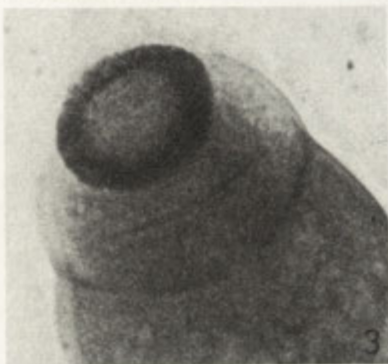
Prytherch H. F. 1938: Life cycle of a sporozoan parasite of the oyster. Science, 88, 451–452.
Schneider A. 1875: Contribution a l'histoire des grégarines des invertébrés de Paris et de Roscoff. Archs Zool. Exp. Gén., 4, 493–604.
Schneider A. 1892: Signalement d'un nouvear sporozoaire. Tabl. Zool., 2, 209–210.
Sprague V. 1949: Species of *Nematopsis* in *Ostrea virginica*. J. Parasit., 35 (6/2), 42.
Sprague V. 1954: Protozoa. In P. S. Galtsoff, ed. Gulf of Mexico. Its Origin, Waters and Marine Life, U. S. Fish Wildl. Serv. Fish. Bull., 55 (89), 243–256.
Théodoridès J. 1961: Sur la distinction entre les grégarines des families des *Cephaloidophoridae* et des *Porosporidae* parasites de crustacés décapodes. C. R. Acad. Sci., 252, 3640–3642.
Théodoridès J. 1962: Grégarines d'invertébrés marins de la region de Banyulus I. Eugrégarines parasites de crustacés décapodes. Vie Milieu, 13, 95–122.
Théodoridès J. 1967: Grégarines d'invertébrés marins de la région de Banyulus IV. Quelques Eugrégarines du genre *Cephaloidophora* parasites de crustacés amphipodés et decapodés. Vie et Milieu, 18, 265–279.
Théodoridès J. 1979: Considerations sur la specificite des Eugrégarines de Brachyoures (*Crustacea Decapoda*). C. R. Acad. Sci., 289, 363–365.
Tuzet O. and Ormières R. 1961: Sur quelques grégarines parasites de Crustacés Décapodes. Annl. Sci. Nat., Zool. 12, 773–783.
Vivárès C. P. 1970: Parasites de crustacés décapodes brachyoures du Golfe et du Lac de Tunis. Bull. Inst. Océanog. Pêchés. Solammbô, 1, 181–203.
Vivárès C. P. 1971: Les grégarines Porosporides parasites des crustacés décapodes brachyoures Mediterranéans. Vie et Milieu, 22, 55–68.
Vivárês C. P. 1978: Grégarinoses et Microsporidioses de Brachyoures (Crustaces Decapodes) de la Méditerranée occidentale: aspects cytologiques, biochimiques et physiologiques (Thése Doct.) Montpellier. 227 + XXI p + annexes.
Vivárès C. P. and Rubió M. 1969: Protozoa parasites de *Crustacea Decapoda Brachyura* de la côte nord-est de l'Espagne. Publ. Inst. Biol. Apl., 4, 111–129.
Watson M. E. 1916: Three new gregarines from marine *Crustacea*. J. Parasit., 2, 129–136.

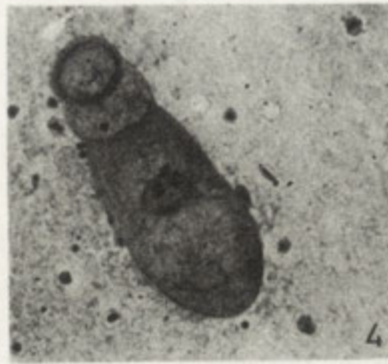
http://rcin.org.pl

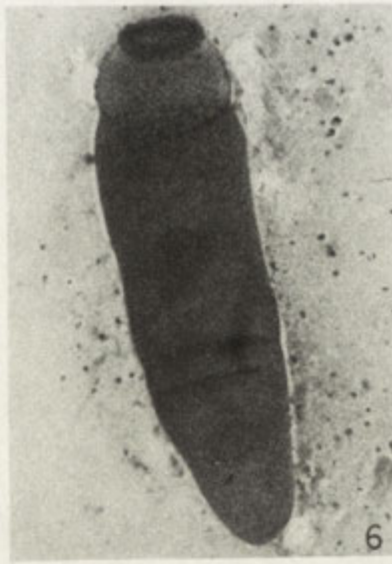
EXPLANATION OF PLATE I

Stephanospora paratelphusae gen, n., sp. n.
1: Biassociative sporadins
2: Triassociative sporadins
3: Anterior region of the primate showing the epimerite
4: Trophozoite
5,6: Early associations

http://rcin.org.pl







S. Prema and K. P. Janardanan auctores phot.

http://rcin.org.pl

http://rcin.org.pl

BOOK RECEIVED

SLEIGH MICHAEL A[LFRED]: PROTOZOA AND OTHERS PROTISTS

[First publ. 1973 as The Biology of Protozoa; This edition first publ. 1989].

Edward Arnold. A division of Hadder and Stoughton. London, New York, Melbourne, Auckland; pp. X, 342; [Price] 20 Ł. ISBN 0-7131-2943-3

CONTENTS

Preface to first edition. Preface to second edition. 1 — Protozoa as members of the Protista. The origins and evolution of protists. Features of the four sets of protistan phyla. The cellular organisation of protists. 2 — Features of protistan organisation. Membranous structures. Fibrous structures. The nucleus. Extracellular products. 3 — Nutrition, metabolism and growth. Nutrition of Protista. Biochemical pathways of metabolism. Feeding mechanisms. Growth. 4 — Reproduction and sex. Nuclear and genetic aspects of reproduction. Division of the cell and morphogenesis. 5 — Flagellates and related protists. Cellular structure. Features of the main phyla. Relationships among flagellated protists and non-flagellated. Algae. 6 — Amoeboid protists. Features of amoeboid groups. 7 — The ciliates. The nuclei of ciliates. Organisation of the ciliate pellicle. Mouth ciliature. Characteristics of different ciliate groups. Ciliate classification and phylogeny. 8 — Spore-forming parasitic protozoa. Phylum *Sporozoa* (= *Apicomplexa*). Phylum *Microsporidia* (= *Microspora*). Phylum *Haplosporidia* (= *Ascespora* in part). Phylum *Myxosporidia* (= *Myxospora*, = *Myxozoa*). 9 — Ecology. Protists in ecosystems. Features of the life of protists in various habitats. Symbiotic relationships involving protists. Appendix. References. Index.

http://rcin.org.pl

NOTICE TO AUTHORS

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

Papers are accepted in English, French, German and Russian. Every paper snould begin with the name and postal address of the laboratory, name and the surname of the author and title in the language of the text. The titles in German and French should be translated into English according to the demands of Current Contents. The paper should be accompanied by synopsis in the language of the text not exceding 100 words and by short summary in one of 4 languages accepted in the Journal. In the Russian text also the name and the postal address of the laboratory, legends of tables, plates and text illustrations must be translated, the translation of the summary may be somewhat more extensive, and the name of the authors should be given additionally also in the Latin characters.

Manuscripts should be doublespaced typescrit (30 lines one side of a sheet) with a normal margin. No elements of the text should be fully typed in capitals nor in spoced set (only underlining with pencil in admittable). In decimal fractions points not commas should be used. The generally accepted abbreviations and symbols are recommended. Nomenclature must agree with the International Code of Zoological Nomenclature, London 1961. The original and two carbon copy of the whole text material should be supplied.

References must be cited in the text indicating only the authors and year thus:

"Kinosita (1954) found that, etc."

Only all references cited in the text should be listed. The list must be arranged as follows:

Ehret C. F. and Powers E. L. 1959: The cell surface of *Paramecium*. Int. Rec. Cytol., 8, 97–133.

Gelei J. von 1939: Das aussere Stutzgerustsystem des Parameciumkorpers. Arch. Protistenk., 91, 245–272.

Titles of references are given in their original language (not translated). In popers written in English, French or German, the Cyrillic type of the Russian in transliterated according to the international system (ISO Recommendation R 9 September 1954). This regulation is not applied to names if there exists in their traditional spelling. Also author may freely chose in transliteration of his own name. In Russian papers, the Russian references are cited in Cyrilic, the others in the Latin characters, but they must be listed all together in the Latin alphabetical order.

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

Galley proofs are sent to the authors. Authors receive 75 reprints without covers.

http://rcin.org.pl

In preparation:

N. Ricci and V. Tortorella: The Ethogram of *Blepharisma japonicum* (*Protozoa, Ciliata*) – M. Krawczyk, M. Gołembiewska-Skoczylas and S. Radzikowski: Cytoplasmic Zones Responsible for Differentiation of the New Macronucleus and Degradation of the Old in *Chilodonella steini* (*Trithigmostoma steini*) – L. Bużańska: Genetic and Epigenetic Factors Controlling Protrusion Occurence in cda A1 Mutant of *Tetrahymena thermophila* – T. Michałowski: The Synthesis and Turnover of the Cellular Matter of Ciliates in the Rumen – L. Kõhidai and G. Csaba: A Functional Study of the Dexamethasone-Induced Steroid in *Tetrahymena* – M. Agarwal: and D. M. Saxena: Effects of Organophosphorus Insecticide Phosphamidon on Ciliate Protozoan *Tetrahymena pyriformis* – A. C. Majewska, W. Kasprzak and M. Błotna-Filipiak: Electron Microscopy of Jejunum of Rats Infected Experimentally with *Giardia muris* – D. Chardez: Notes sur *Cyphoderia perlucidus* Beyens et Chardez, 1986 et sur le periphyton des plantes aquatiques – G. Poglayen, M. Catani and G. Battelli: *Eimeria* spp. of roe deer (*Capreolus capreolus* L.) in an Apenninic Area of Italy

Warunki prenumeraty

Prenumeratorzy indywidualni, instytucje i zakłady pracy zamawiają prenumeratę w Oddziałach RSW „Prasa-Książka-Ruch" właściwych dla miejsca zamieszkania lub siedziby prenumeratora.

Prenumeratę ze zleceniem wysyłki za granicę przyjmuje RSW „Prasa-Książka-Ruch", Centrala Kolportażu Prasy i Wydawnictw, ul. Towarowa 28, 00-958 Warszawa, konto PBK XIII Oddział Warszawa Nr 370044-1195-139-11.

Prenumerata ze zleceniem wysyłki za granicę pocztą zwykłą jest droższa od prenumeraty krajowej o 50% dla zleceniodawców indywidualnych i o 100% dla zlecających instytucji i zakładów.
Na życzenie prenumeratora dostawa może odbywać się drogą lotniczą; koszty dostawy lotniczej w pełni pokrywa prenumerator.

Terminy przyjmowania prenumeraty:

I półr. i cały rok następny	– na kraj do 10 XI
	– na zagranicę do 31X
na pozostałe okresy prenumeraty:	do 1 dnia każdego miesiąca poprzedzającego okres prenumeraty roku bieżącego

Bieżące i archiwalne numery można nabyć lub zamówić we Wzorcowni Ośrodka Rozpowszechniania Wydawnictw Naukowych PAN, Pałac Kultury i Nauki, 00-901 Warszawa.

http://rcin.org.pl

Cena 480 zł

CONTENTS

L. Kuźnicki: Jan Dembowski (1889-1963) 213
L. Kuźnicki and P. L. Walne: The Relationship Between Step-up and Step-down Photophobic Responses in *Euglena gracilis* 215
J. Fyda: La Morphologie et Stomatogenese de *Spathidium amphoriforme* (Greff, 1888) (*Ciliophora*: *Kinetofragminophora*) [Morphology and Stomatogenesis of *Spathidium amphoriforme* (Greeff, 1888) (*Ciliophora*: *Kinetofragminophora*)] 231
A. Sola, A. Guinea and D. Fernandez-Galiano: Light Microscopical Study in the Conjugation Process in *Opisthonecta henneguyi* Fauré-Fremiet (*Ciliophora*: *Peritrichida*) 239
W. Krawczyńska, N. N. Pivovarova and A. Sobota: Effects of Cadmium on Growth, Ultrastructure and Content of Chemical Elements in *Tetrahymena pyriformis* and *Acathamoeba castellanii*: 245
H. Fabczak and S. Fabczak: Modulators on Intracellular Sodium Concentration Affect Contractile Vacuole Activity in Ciliate *Blepharisma japonicum* 253
S. Fabczak and H. Fabczak: Application of Ion-Selective Microelectrode in Measurement of Intracellular Chloride Activity in Ciliate *Blepharisma japonicum* 265
T. Michałowski, I. Landa and P. Muszyński: Factors Influencing the Development of Population of the Rumen Ciliates *Anoplodium denticulatum* in Vitro 273
W. Kasprzak, W. Golec and E. Hadaś: Epizootics of Reptilian Amoebiasis at the Zoological Gardens of Poznań (Poland); Epidemiological and Chemotaxonomic Studies 285
K. Ramanandan Shanavas, P. Ramachandran and K. P. Janardanan: *Trypanoplasma ompoki* sp. n. from Freshwater Fishes in Kerala, India, with Observations on its Vector-Phase Development and Transmission 293
S. Prema and K. P. Janardanan: Observations on *Stephanospora paratelphus* gen. n., sp. n. (*Apicomplexa*: *Cephalina*) from the Freshwater Crab, *Paratelphusa hydrodromus* (Herbst) 303
Book Received 311

Państwowe Wydawnictwo Naukowe – Oddział we Wrocławiu

Wrocławska Drukarnia Naukowa

Indeks 351334

http://rcin.org.pl