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Review article

# *Stentor* and *Blepharisma* Photoreceptors: Structure and Function

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**Summary.** *Stentor coeruleus* and *Blepharisma japonicum* are single cell ciliates, and yet they can detect light with high efficiency, exhibiting well-defined photophobic responses. The photoreceptors that are responsible for the light-avoiding behavior have been identified as stentorin and blepharismin (or oxyblepharismin) respectively. The structure of stentorin was determined to be octahydroxydiisopropylnaphathodianthrone, and that of blepharismin, although not fully elucidated, is also a hypericin analogue. The photochemistry of this class of novel photoreceptors is unknown; however, proton and/or electron transfer may play a significant role in the primary photoprocess. G-proteins and phosphodiesterase appear to be involved in the sensory signal transduction and amplification.

**Key words**. *Stentor*, *Blepharisma*, stentorin, blepharismin, G-protein, sensory transduction.

## INTRODUCTION

Greenish-blue colored *Stentor coeruleus* and pink-colored *Blepharisma japonicum* are unicellular ciliates. They tend to accumulate in shady or dark areas (Holt and Lee 1901; Jennings 1904; Mast 1906; Giese 1973, 1981). They swim freely under normal conditions. However, when a light trap is encountered, they stop and even swim backwards momentarily. *Blepharisma* also rotates briefly around an axis perpendicular to its swimming direction and then swim forwards in a new direction (Fig. 1). These light induced responses were further characterized as a step-up photophobic and a negative phototactic response (Song et al. 1980a, b; Scevoli et al. 1987; Matsuoka et al. 1992a; Checcucci et al. 1993; Gioffre et al. 1993). Additionally, *Stentor coeruleus* showed a negative photokinesis (Iwatsuki 1991), and *Blepharisma japonicum* showed a step-down photoresponse (Matsuoka and Taneda 1992).

It is remarkable that the small single-cell aneural motile organisms have such high photosensitivity. As in any other stimulus-response system, light stimulus in these ciliates is perceived by their intrinsic receptors; namely photoreceptors which absorb the light of a particular wavelength. Based on the action spectra for the photophobic response and for the light-induced bioelectrical responses, stentorin in *Stentor* and blepharismin in *Blepharisma,* have been identified as the photoreceptor molecules that are responsible for the light avoiding behavior in these organisms (Wood 1976; Song et al. 1980a, b; Song 1981, 1983; Scevoli et al. 1987; Matsuoka et al. 1992a; Checcucci et al. 1993). These photosensor molecules are imbedded just beneath the pellicle in the

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pigment granules which are arranged longitudinally along the cell body parallel to the ciliary rows.

Interestingly, *Blepharisma japonicum* changes its color from red to blue upon light irradiation at moderate intensity. The blue-turned cell also shows photophobic response with oxyblepharismin as its photosensor molecule. The photoreceptor molecules, stentorin as well as blepharismin, have been proposed to be hypericin-like based on the similarities in spectroscopic characteristics. These hypericin analogues represent a novel class of photoreceptor molecules with a significantly different chromophore structure than the known photoreceptors; such as rhodopsin, bacteriorhodopsin, chlorophylls, phytochrome, and flavins. Investigations of the photosensory transduction in these ciliates would shed light on our understanding of the mechanistic nature of stimulus-response systems. *Stentor coeruleus* and *Blepharisma japonicum* are particularly suitable for this purpose since they are unicellular and the problems associated with complex interactions that occur between mutually interdependent cells in higher organisms can be avoided. In addition, *Stentor coeruleus* is one of the largest ciliates. Its typical size is 700 μm in length. *Blepharisma japonicum* is not quite as large and has a typical size of 400 μm in length. Both are deeply pigmented and readily visible to naked eyes.

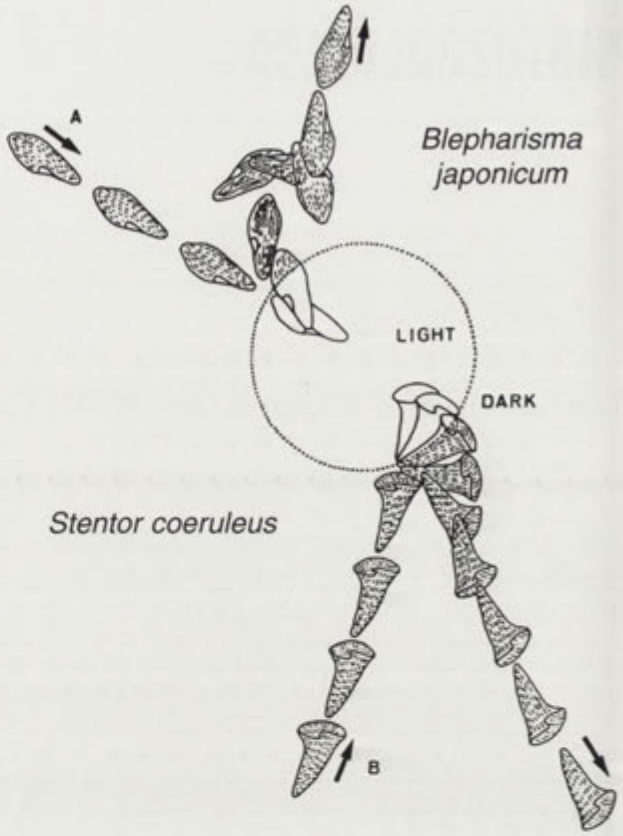



Fig. 1. Schematic drawing of the photophobic responses of *Stentor coeruleus* (B) and *Blepharisma japonicum* (A)

## CHROMOPHORE STRUCTURE

The pigment of *Stentor coeruleus* has been of interest for many years. Its investigation dates back to 1873 when Ray Lankester named it stentorin (Lankester 1873). Later, a thorough chemical and spectroscopic analysis of the pigment by Møller led him to conclude that stentorins belonged to the meso-naphthodianthrone class of compounds (Møller 1962). In 1979 Edward Walker proposed that the native chromophore was linked to a 65 kD protein and that the released chromophore upon acid hydrolysis was hypericin (Walker et al. 1979). However, he failed to notice that even the absorption maximum of acid treated stentorin was not entirely identical to that of hypericin at low pH's. In fact, stentorin, which was HPLC-purified from a crude extract of *Stentor coeruleus* cells, has a $\lambda_{max}$ at 595 nm (in methanol), while hypericin has one at 588 nm (Tao et al. 1993). When acidified, the $\lambda_{max}$ for stentorin and hypericin shifted to 577 nm and 578 nm, with somewhat different molar extinction coefficients at the absorption maxima, respectively.

In negative ion fast atom bombardment (FAB) mass spectrometry (MS), stentorin showed an $(M\text{-}1)^-$ ion at 591.1304, which is in accord with the $(M\text{-}1)^-$ of $C_{34}H_{24}O_{10}$ ($(M\text{-}1)^-$ calculated to be 591.1291). Results from acetylation of stentorin, in combination with FAB-MS, indicated that there are eight hydroxyl groups in stentorin. NMR spectrum of stentorin revealed the presence of isopropyl groups in stentorin. Thus the structure of stentorin has been narrowed to two possible symmetrical isomers, **1** and **2** (Fig. 2). Further studies showed structure 1 to be the native chromophore (D. W. Cameron, personal comm., and unpublished CD results from this Laboratory). However, whether or not the chromophore in the native pigment stentorin II (see below) is the same as the isolated chromophore is still unknown.

The pigment of *Blepharisma* was studied beginning early in this century by Arcichovskij (1905). He studied the ethanol extract of the pigments from *Blepharisma laterilium* and named it zoopurpurin. Emerson (1930) and Giese (1953) investigated the solubility and absorption maximum of the pigments from an American

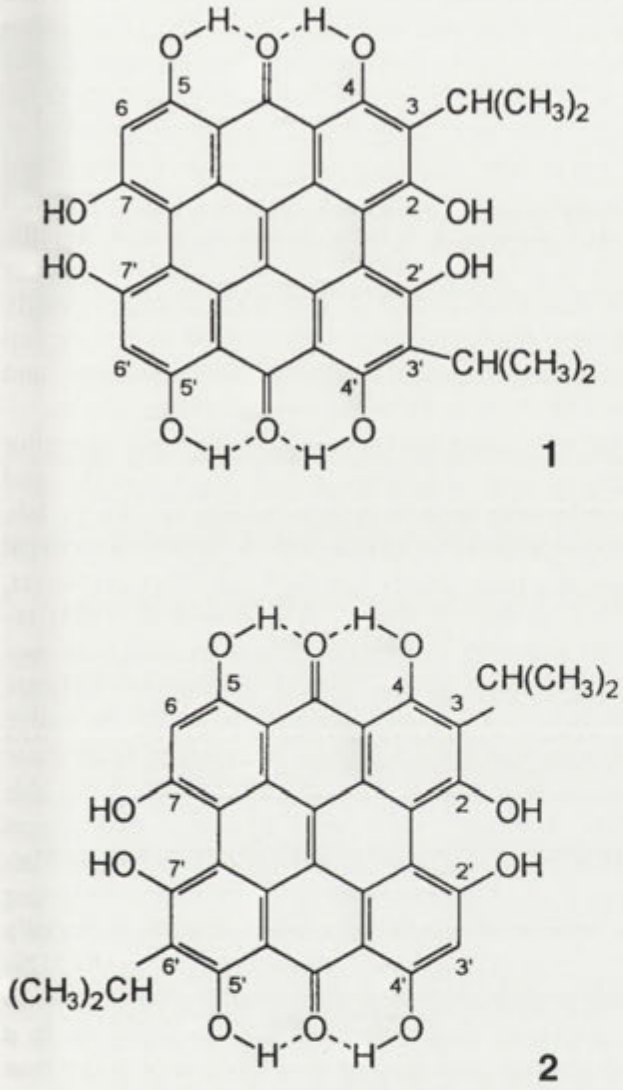



Fig. 2. Possible structures of stentorin

species of *Blepharisma undulans* in various solvents. Møller (1962) showed that the pigment of an American species of *Blepharisma undulans* strongly resembled hypericin in absorption maxima. In 1965 Sevenants carefully analyzed the pigment of *Blepharisma undulans* and reported that the pigment possessed the same mesodianthrone structure as hypericin (Sevenants 1965). In the early 1970's Giese carried out a series of studies on the pigments from *Blepharisma americanum*, *Blepharisma intermedium* and *Blepharisma japonicum* and other species (Giese 1973, 1981). He found that the pigments exist in red and blue forms in all species tested. They were also essentially alike in spectrophotometric and solubility properties as well as in their sensitivity to light (Giese and Grainger 1970). Due to the very limited amount of material, the pigment of *Blepharisma* has not been purified and its structure has not been elucidated chemically. However, recently we have accumulated enough *Blepharisma japonicum* cells and the preliminary study of the HPLC-purified pigments from the cell extract showed that the red to blue transformation of blepharismin occurred via a loss of two mass units. Detailed results will be reported in a separate paper.

## PHOTORECEPTOR PROTEINS

Two groups of pigments, stentorin I and stentorin II, exist in *Stentor coeruleus* (Møller 1962). Stentorin I was extracted with ethanol. Stentorin II, which was insoluble in ethanol, was obtained by extraction with acetone/water (80/20), after stentorin I was exhaustively extracted with ethanol. Stentorin I, and stentorin II, had similar spectral absorption properties. However, stentorin I was strongly fluorescent while stentorin II was only weakly or non-fluorescent (Møller 1962). In 1978 Walker named the organic solvent-soluble pigment, stentorin I, and the protein-bound pigment, stentorin II (note the difference between Walker's and Møller's classification). In his report Walker stated that stentorin I was readily extracted by mildly shaking the cell suspension in ethanol, acetone or n-pentane. Stentorin II was obtained by sonication of a cell suspension in phosphate buffer, followed by ammonium sulfate fractionation and sucrose density gradient centrifugation. Although he pointed out the differences between stentorin I and hypericin in their solubility and partition coefficients, it was also claimed that stentorin I was identical to hypericin after acid hydrolysis. Four bands in SDS PAGE gels were reported for stentorin II (Walker et al. 1979). In 1988 Kim purified two pigment fractions from *Stentor coeruleus* by fractionating the crude buffer extract with column chromatography (Kim et al. 1990). He also used the terms stentorin I for the highly fluorescent fraction and stentorin II for the non-fluorescent one. Although somewhat different than Walker's classification, Kim's was basically consistent with Møller's in terms of their fluorescence. It should be noted that a neutral detergent, reduced Triton X-100, was included in Kim's buffer solution to increase the solubility of membrane-bound stentorin II. Partial purification of stentorins was performed by using a hydroxyapatite (HA) column and a Bio-Gel A - 1.5m gel filtration column (Kim et al. 1990). Stentorin I ($\lambda_{max}$ at 608 nm, strongly fluorescent) appeared to be a relatively small complex composed of at least two

heterologous subunits corresponding to apparent molecular weights of 46 kD (fluorescent, Coomassie blue negative) and 52 kD (fluorescent, Coomassie blue positive) on 13% SDS-PAGE gels. Stentorin II ($\lambda_{max}$ at 620 nm, weakly or non-fluorescent) was probably a large protein complex which was eluted out of the Bio-Gel A - 1.5m column near the void volume.

Both stentorin I and II displayed anomalous behaviors on SDS-PAGE gels. For example, the molecular weight of stentorin I was strongly dependent on the gel concentration and one of the lower bands was a rather smeared band. Stentorin II did not enter the gel. These anomalies presented the possibility that stentorins were probably glycosylated since the dependence of molecular weight on gel concentration is quite characteristic of glycoproteins (See and Jackowski 1989). Later it was reported that both stentorin I and stentorin II exhibited positive reactions with biotinylated Concanavalin A (Con A) which is specific for sugars containing terminal glucosyl and mannosyl residues. Only stentorin II exhibited a positive reaction with biotinylated wheat germ agglutinin (WGA), which is specific for N-acetylglucosamine residue (Kim et al. 1990). However, a recent study showed that neither stentorin I, nor stentorin II, reacted with lectins including Concanavalin A (Con A), wheat germ agglutinin (WGA), pea lectin, E-PHA, L-PHA etc. This result was confirmed since neither stentorin I nor stentorin II could be stained (Tao and Song, unpublished result) with Glycan Differentiation Kit from Boehringer Mannheim Biochemica (Indianapolis, IN). It seems unlikely that stentorin I and stentorin II are normal glycoproteins, although these qualitative tests cannot be considered unequivocal.

Since stentorin II exhibits an ultrafast photoprocess, and stentorin I does not, it was proposed that stentorin II may serve as the functional photoreceptor (Song et al. 1990). Recently, stentorin II-B was isolated from stentorin II by hydrophobic interaction chromatography (Dai 1994). It contains the chromophore covalently bound to an approximately 50 kD protein, as determined by SDS-urea-PAGE (Dai 1994). Further characterization of stentorin IIB is in progress in this lab. Stentorin I and stentorin II, in their native states within the *Stentor* cell, are spectrophotometrically indistinguishable and the relation between them in the pigment granules remains elusive (Kim et al. 1990). However, a photoacoustic spectroscopic study clearly demonstrated the existence of the two forms of the pigment in the cell (reported later in this paper).

It is interesting that *Blepharisma japonicum*, when exposed to light of moderate intensity, turns from red to blue (Giese 1981). Both red- and blue-pigmented cells show step-up photophobic responses (Matsuoka et al. 1992a, Checcucci et al. 1993). The action spectrum of the response in red cells closely resembles the absorption spectrum of the red pigment, while the action spectrum of the response in blue cells closely resembles the absorption spectrum of the blue pigment (Matsuoka et al. 1992a, Checcucci et al. 1993). This finding strongly suggests that blepharismin is involved in the step-up photophobic response in *Blepharisma japonicum*, and rules out a rhodopsin as the possible photoreceptor.

In an attempt to isolate the native photoreceptor pigment from *Blepharisma japonicum*, Gioffré and coworkers in Lenci's group, and Dai in Song's lab, isolated a presumed photoreceptor protein of apparent molecular mass 38 kD (Gioffré et al. 1993). However, Matsuoka and coworkers (Matsuoka et al. 1993) reported that SDS-PAGE, of a major chromophore-containing fraction eluted from a gel-filtration column, indicated a 200 kD chromoprotein to be the native photoreceptor. Further work is necessary to verify these contradicting results. It should be mentioned that different detergents were used by these two groups (CHAPS by Gioffré et al. and sodium cholate by Matsuoka et al.). Caution should be exercised in determining the molecular weight of chromoproteins (especially those containing hypericin-like chromophores) by SDS-PAGE or gel-filtration chromatography, since stentorin chromophore (molecular weight 592) dissolved in a detergent-containing buffer may appear to be bigger than 100 kD (Tao and Song, unpublished results).

## PIGMENT GRANULE AND LOCALIZATION

The pigment granules in *Stentor coeruleus* are 0.3-0.7 μm in diameter and are distributed longitudinally along the cell body between ciliary rows (Huang and Pitelka 1973). They were believed to situate near the ciliary basal bodies fused into the pellicle, or plasma membranes (Kennedy 1965, Newman 1974). A recent scanning electron micrograph study showed that pigment granules were packed as close as possible in a random manner within each of their rows. A transmission electron micrograph study revealed that the pigment granules were striated with alternating layers having high and low electron density; nonetheless, amorphous pigment granules were also evident. It is uncertain if the

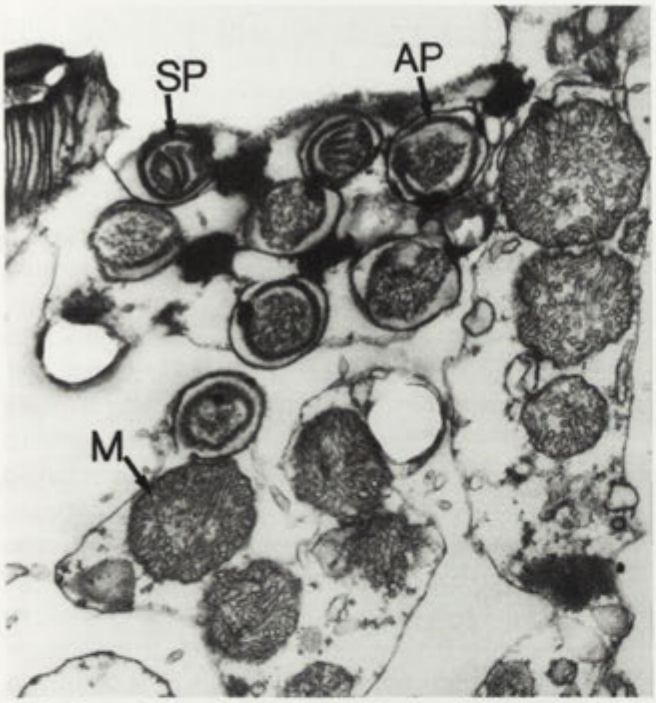



Fig. 3. Transmission electron micrographs showing both striated (SP) and amorphous (AP) pigment granules. Mitochondria (M) are also seen on the lower part and upper right-hand side of the photo

amorphous appearance of the pigment granule resulted from artifactual loss of the striated fine structure. No mitochondrial fine structure was observed in the pigment granules (Newman 1974, Small and Marszalek 1969), although the pigment granules were thought to be mitochondria-like (Weisz 1949, 1950). As a matter of fact, Meza-Keuthen (Meza-Keuthen 1992) observed a distinct structure of mitochondria (Fig. 3), consistent with an earlier observation (Randall and Jackson 1958).

## SPECTROSCOPY OF THE PIGMENTS *IN VIVO*

Stentorin I and stentorin II, in their native states within the *Stentor* cell, are spectrophotometrically indistinguishable and the relation between them in the pigment granules has not been established (Kim et al. 1990). To test the possible existence of two chromoproteins in a native *Stentor* cell, photoacoustic spectroscopy was applied. Photoacoustic spectroscopy is specially applicable to non-transparent and highly scattering media (Braslavsky 1986). The photoacoustic signal involves the absorption of modulated light by the sample. Radiationless deactivation processes produce heat, which is distributed to the sample surface. This results in a modulated pressure change, detected by a microphone (Rosencwaig 1980). The ability of the photoacoustic spectroscopy to determine *in vivo* spectra of biological species (O'Hara et al. 1983), to study morphology of plant tissues (Farringer et al. 1985), or detection of electronic transition bands that are hidden in absorption spectra (Pottier et al. 1988), is well established. Photoacoustic spectroscopy proved to be useful in studying the excitation energy path in biological systems (Frackowiak et al. 1985, Romanowski et al. 1987). Therefore photoacoustic spectroscopy is a well suited tool for experimental study of pigmented ciliate cells *in vivo*.

Photoacoustic spectra can be measured using a single beam photoacoustic spectrometer (PAS). A light source for a PAS is a 1000 W xenon arc lamp or alternative source including tunable laser. Light from the monochromator is mechanically chopped and focused into the photoacoustic cell, which is a gas-microphone system based on a design described by Ducharme et al. (Ducharme et al. 1979). In the PAS system, constructed in the author's laboratory, the frequency of light modulation is 26 Hz. The incident monochromatic light flux is 3 $mW/cm^2$ at 550 nm. The photoacoustic signal is collected by a ceramic microphone, equipped with a preamplifier. Amplified and filtered signal is converted by an analog-to-digital converter, and processed by a personal computer. The photoacoustic spectra are corrected for the spectral output of the excitation system. Contribution to the photoacoustic signal from the filtration paper, used as a support for the sample, was flat through the light wavelength being used.

We developed a computer-based method of photoacoustic signal analysis. We incorporated some of the concepts for photoacoustic data analysis, as presented by Anjo and Moore (1984). Our method replaces a standard lock-in amplification. The signal from a microphone is processed to obtain both amplitude and phase. This signal $A(\lambda,t)$, converted by analog-to-digital converter, is a function of wavelength $\lambda$ and time t as the signal modulated with a frequency $f_M$ is time dependent. The signal is sampled with a frequency $f_S = f_M \times 256$ over a period of time $T = 1/f_M$. This measurement is repeated N times, where N is a user-defined number, and then averaged:

$$A(\lambda,t) = 1/N \, \Sigma_i \, A_i(\lambda,t),$$

where t runs over a single cycle of the light modulation $T=1/f_M$, N=20, and $\lambda$ is fixed during the time of this repetitive measurement.

Two phase resolved components, sine and cosine, are calculated as follows:

$$S(\lambda) = {}_T\int[A(\lambda,t) \times \sin(2\pi \times f \times t)]\, dt,$$
$$C(\lambda) = {}_T\int[A(\lambda,t) \times \cos(2\pi \times f \times t)]\, dt, \quad (1)$$

where S and C are, respectively, sine and cosine components of the analyzed photoacoustic signal A. The sine and cosine components of photoacoustic signal are the original data obtained from this kind of signal processing. The two components plotted against a wavelength of exciting light actually represent time-resolved spectra, where the time resolution corresponds to a phase lag between the cosine and sine functions. Therefore, these two components correspond to "fast" and "slow" processes of thermal deactivation. At the modulation frequency being used in these experiments (26 Hz), the time-lag between cosine and sine components is equal to 10 ms.

A resultant vector of the photoacoustic signal is calculated as:

$$R(\lambda) = [S(\lambda)^2 + C(\lambda)^2]^{1/2}. \quad (2)$$

Phase shift of photoacoustic signal, representing a time-lag of the thermal deactivation associated with different parts of the photoacoustic spectrum, can be obtained as:

$$\Phi(\lambda) = \arccos[C(\lambda)/R(\lambda)]. \quad (3)$$

The depth into the sample probed by the photoacoustic technique may be approximated as a thermal diffusion length $\mu_T$ (Rosencwaig 1980):

$$\mu_T = (2\alpha/\omega)^{1/2}, \quad (4)$$

where $\alpha$ is thermal diffusivity of the sample, and $\omega = 2\pi \times f_M$ where $f_M$ is the frequency of light modulation. Thermal diffusivity of most biological samples is ca. 0.1 $mm^2/s$ (Touloukian et al. 1973). At the frequency $f_M$ = 26 Hz being used, the depth of penetration is approximated as $\mu_T = 35\ \mu m$, which is an appropriate value for a morphological study of the *Stentor* cell, whose diameter does not exceed 100 µm. This kind of analysis shows individual contributions to the absorption from different depths and is specially useful in analyzing heterogenous systems (O'Hara et al. 1983). Some knowledge of the structure being investigated is helpful in the spectral data interpretation.

The cosine and sine components of the photoacoustic signal shown in Fig. 4 are characterized by different positions of photoacoustic signal band in the red part of spectrum (around 600-620 nm). The red band of sine component is located at 603 nm, whereas the corresponding band of cosine component is shifted 11 nm toward longer wavelengths. This indicates that there are two light-absorbing species, distinguishable by a measurement of the photoacoustic signal at different time lags between excitation pulse of light and thermal deactivation process. The cosine and sine components presented in Fig. 4 are two different combinations of photoacoustic signals originated from these species. Certainly, it would be interesting to resolve or decompose the cosine and sine components into separate photoacoustic signals related exclusively to either one of these light absorbing species. Unfortunately, as seen in Fig. 4, these spectral components strongly overlap, which makes a unique decomposition rather impossible. Therefore, we conclude that both, cosine and sine components of photoacoustic signal, originate from two species or chromophores having similar spectral characteristics. One of these chromophores, which is present mainly in the sine component (characterized by a longer time lag), has the maximum of photoacoustic signal shifted toward shorter wavelengths, and the other, prevailing in the cosine component (having shorter time lag), has the maximum of photoacoustic signal shifted toward longer wavelengths.

The resultant photoacoustic spectrum shown in Fig. 5 is calculated according to equation (2). This spectrum represents some of the important characteristics that are known from the phase-resolved components (Fig. 4). The main maximum in the red part of the resultant photoacoustic spectrum is located at 612 nm with accompanying shoulder at short-wavelength slope of the band (566 nm). Moving toward shorter wavelengths, this band is followed in the blue region by a less structured part of the spectrum (380-480 nm) and a strong band in the near-UV (350 nm). These three bands (red, blue and near UV), as described for the resultant photoacoustic spectrum, are also present in its sine and cosine components (Fig. 4), as well as in the absorption spectrum of pigment granules (Kim et al. 1990). In the latter the 612 nm maximum is shifted 6 nm toward the longer wavelengths (red-shifted). Interestingly, a ratio of the signal intensity in the red to that in the near-UV has very close value in both absorption and photoacoustic spectra, and equals approximately 0.8. On the other hand, a ratio of the signal intensity in the red to that in blue is 0.9 for photoacoustic spectrum, and increases to 1.8 for absorption spectrum (Kim et al. 1990). Therefore, there is a strong tendency for a decrease in intensity of the blue

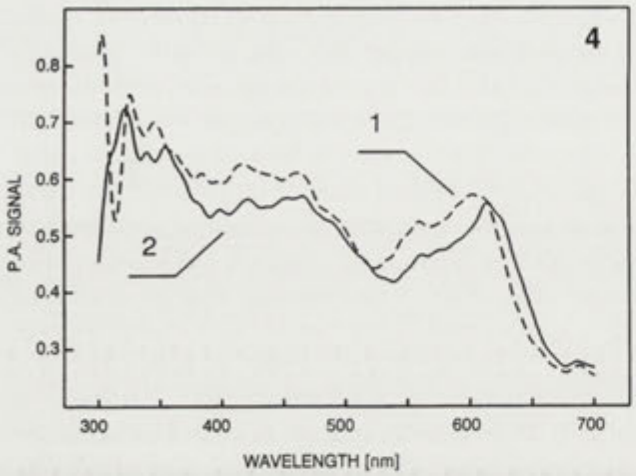
band, relative to the red and near-UV, in going from native cells to pigment granules. This tendency for relative intensity decrease in the blue part of spectrum, accompanied by more conserved ratio of intensities in the red to that in the near-UV, is even more profound when the comparison includes the stentorin I and stentorin II absorption spectra (Kim et al. 1990). The relative intensity of the blue part of spectrum (380-480 nm) may serve as an inverse indicator of purity of the isolated chromophores.

A ratio of the photoacoustic signal to that of absorption has been used to measure the efficiency of thermal deactivation as one of competing deactivation processes (Frackowiak et al. 1985, Romanowski et al. 1987). Due to the high optical density it is impossible to obtain this ratio directly for *Stentor* cells. Therefore, we compared the photoacoustic spectra of whole cells to the absorption spectrum of pigment granules (Kim et al. 1990). The 6 nm blue-shift of photoacoustic spectrum relative to that of absorption (see paragraph above) indicates that the photoacoustic-to-absorbance signal ratio, and consequently the thermal deactivation signal, is higher at the short-wavelength slope of absorption band and is lower at the long-wavelength slope of that band. Different experimental conditions, which include sample preparation and monochromator slits, do not allow a quantitative analysis of the thermal deactivation signal. Therefore, we estimated a possible effect of the observed spectral shift on magnitude of the thermal deactivation signal as follows. Assuming that the shape of the photoacoustic band is the same as that of absorbance, and using the experimentally detected shift of positions of these bands (6 nm), we calculated that the thermal deactivation signal may change from 2.2 to 1 going from the short- to long-wavelength slope of absorption band. For light-driven processes, low thermal deactivation signal usually indicates high efficiency of that process (Frackowiak et al. 1985, 1990). Therefore, we conclude that the chromophore absorbing at the longer wavelength is involved in a more efficient process of energy transfer, or photochemical process, as compared to the chromophore absorbing at the shorter wavelength, where thermal dissipation of absorbed energy prevails.

The presence of two different photoreceptors in the native *Stentor* cells is also evident from a phase shift associated with the resultant photoacoustic signal (Fig. 5). This phase shift, calculated according to equation (3), exhibits a biphasic behavior in the red part of spectrum, around the characteristic band with a maximum at 612 nm. At the short-wavelength slope of this band (i.e., between 540-600 nm) the phase shift equals 44°. At the long-wavelength slope the phase shift gradually decreases to 35°, and is constant between 630-650 nm. At wavelengths shorter than 540 nm, or longer than 650 nm, the phase shift appears to be between those values. The phase shift is related to a time lag between excitation pulse of light (as generated by a chopper) and thermal deactivation process, as detected by microphone (Rosencwaig 1980). This time lag may be correlated with a heterogenous structure or with energy storage processes occurring in the sample, or both (Anjo and Moore 1984, Boucher and Leblanc 1985, Carpentier et al. 1984). We estimated that the observed difference in phase shift corresponds to a 1ms time lag, which may result from as low as 1 μm difference between position of two different chromophores, measured as a depth into sample. Experimental data available do not support the hypothesis that the photoreceptor of *Stentor* is capable of long-time energy storage.

Specifically, study of fluorescence intensity decay of stentorin I and stentorin II does not indicate a presence of the long-lived (i.e., ca. 1 ms) meta-stable state (Song et al. 1990). Therefore, we assume that the observed phase lag of the short-wavelength slope of the red band of photoacoustic signal is related strictly to a morphological characteristics of the *Stentor* chromoproteins. The calculated depth difference is an average value and has to be carefully considered. Because the short-wavelength slope of the red band is in a region where contributions from both chromophores strongly overlap, the phase shift in this spectral region is also affected by this overlapping. Hence, the upper limit of phase shift (44°) do not result from a homogenous chromophore contribution. However, the situation is much better on the long-wavelength slope of the red band, where the red-shifted component presumably dominates. Nevertheless, the indicated depth difference obviously underestimates the average displacement between these chromophores.

The biphasic nature of the photoacoustic signal arises from two forms of pigments that are present in the native *Stentor* cell. We propose that these pigments correspond to those isolated previously, namely stentorin I and stentorin II. According to this postulate, stentorin II has a red-shifted photoacoustic signal maximum and is located preferentially closer to the outer surface of *Stentor* cell. Also, this chromoprotein is involved in a more efficient process of the photochemistry, as compared to stentorin I. On the other hand, stentorin I, which has a blue-shifted maximum of photoacoustic signal, is buried



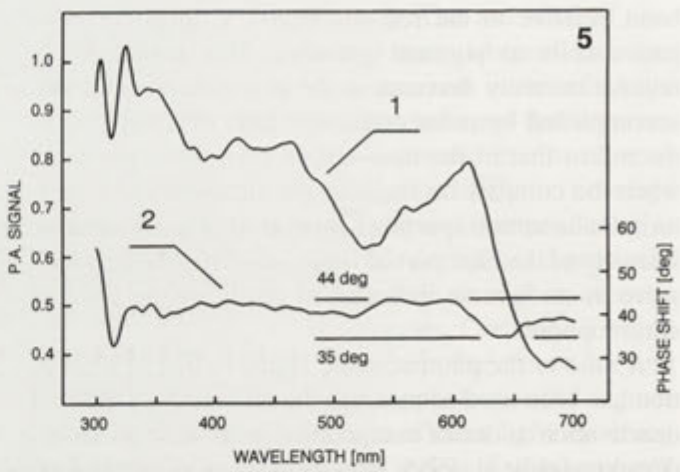




Fig. 4. Cosine (1) and sine (2) components of the photoacoustic spectrum of *Stentor coeruleus*. Intensity of photoacoustic (P.A.) signal in arbitrary units. *Stentor* cells were grown as described previously (Walker et al. 1979). Harvested cells were separated from contaminants using a mesh and then carefully deposited on a filtration paper, 12 mm diameter, to form a possibly homogenous layer of cells. An excess of the liquid culture medium was removed. Each sample was prepared directly before the measurement and placed in the photoacoustic chamber

Fig. 5. Resultant photoacoustic spectrum (1) of *Stentor coeruleus* and its phase shift (2). Intensity of photoacoustic (P.A.) signal in arbitrary units

in the cell. Stentorin I is photochemically less efficient. Because of the strong spectral overlap, as seen in Fig. 4, separate spectral characteristics of those two species cannot be fully determined. For example, we cannot determine the exact positions of photoacoustic signal maxima corresponding to either stentorin I or stentorin II, although it is obvious that the maximum of the photoacoustic red band is shifted toward shorter wavelengths as compared to that band in the absorption spectrum.

The postulated link between the biphasic characteristics of photoacoustic spectra and two forms of pigments, stentorin I and II, is supported by observation of the *Stentor* cells under fluorescence microscope. Fluorescence of the cells originates from the inner part of cell, whereas the outer surface is not fluorescent (Łazowski K. and Song P.-S., unpublished results). The stronger fluorescence indicates less efficient photochemistry processes. Fluorescence of the isolated stentorin I and stentorin II also confirms this assignment. Stentorin I, which is the postulated buried chromophore, is fluorescent, whereas fluorescence of stentorin II is weak (Song et al. 1990). Nevertheless, one has to be careful in extrapolating the information that is obtained from isolated components to a living organism. It is known that the fluorescence of whole *Stentor* cells is much different than that of isolated components. The native cells exhibit large red shift of fluorescence spectra, as compared to the isolated structures (Walker et al. 1981, see later). On the other hand, the postulated assignment of the chromophores detected using photoacoustic spectroscopy is consistent with functional aspects of stentorin I and II, discussed previously (Kim et al. 1990, Song et al. 1990). It has been suggested that stentorin II is the primary photoreceptor (Kim et al. 1990) and this suggestion has been confirmed by the extremely low fluorescence yield of stentorin II (Song et al. 1990). Photoacoustic spectroscopy has also shown that thermal deactivation of the excited states of stentorin II is low compared to that of stentorin I. Since an increase in the thermal deactivation signal often accompanies denaturation, or dissociation of macromolecular complexes (Romanowski et al. 1987, Skibinski et al. 1990), we suggest that stentorin I is a denaturated form of stentorin or free chromophore. Stentorin II does not generate stentorin I under denaturating conditions (Kim et al. 1990).

Summarizing this section, photoacoustic spectroscopy has demonstrated that there are indeed two light absorbing species *in vivo* (Fig. 5). One is presumably closer to the outer cell membrane and its photoacoustic spectrum is red-shifted. Stentorin II is localized within the pigment granules that are distributed between the ciliary rows (see the microspectrophotometry section later). The other, buried into the cell volume, is contributing to energy dissipation; therefore, it is assumed to be less efficient photochemically. Their spectral characteristics are similar to those of isolated pigment

| peak (nm) | Abs. |
|---|---|
| 513 | 0.634 |
| 537 | 0.849 |
| 549 | 0.618 |
| 564 | 1.033 |
| 581 | 0.755 |
| 596 | 0.784 |
| 609 | 0.953 |
| 619 | 1.051 |
| 653 | 0.524 |

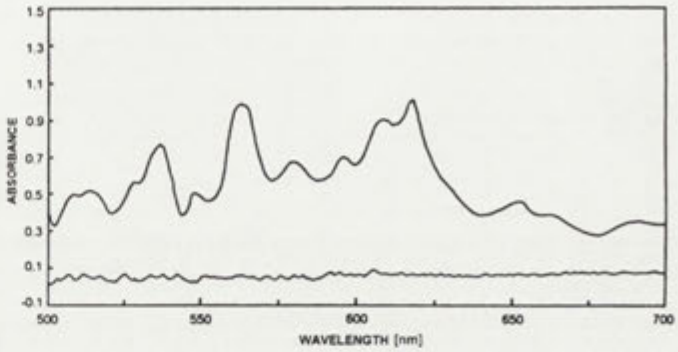



Fig. 6. In situ absorption spectrum of *Stentor coeruleus* taken at the oral part (7.5 µm sampling spot as indicated on the picture)

species, stentorin I and stentorin II (Kim et al. 1990). This may indicate that the red-shifted component, detected in the native cell by photoacoustic spectroscopy, corresponds to stentorin II and the blue-shifted component belongs to stentorin I. According to the presented interpretation of photoacoustic data, stentorin II is preferentially located at the outer membrane and involved in a more efficient process of the utilization of absorbed energy of light.

As is the case with photoacoustic spectroscopy, microspectrophotometry (on intact samples) has the advantage of preserving the integrity of biological structures, and allowing reliable *in vivo* spectroscopic investigation of pigment distribution and composition (Gualtieri et al. 1989, Schmidt et al. 1990). Measurement of absorption spectra of *Stentor in vivo* in a culture medium is difficult because of the negative phototactic response of the organism to visible light. Therefore it was necessary to increase the viscosity of the medium to keep *Stentor* stationary without killing the organism. Egg white proved suitable for this purpose when mixed in a ratio 3:1 with buffer (final conc. 1 mM Tris-HCl, 1 mM $NaNO_3$, 1 mM $KH_2PO_4$, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, pH 7.8), stirred for 20 min and centrifuged (10.000g /10 min) to remove insoluble matter. The supernatant was used for resuspension of *Stentor coeruleus* for microscopic observation and spectrophotometry.

We recorded absorption spectra with a visible- and infrared-light microspectrophotometer (Schmidt et al. 1990). The set-up consists of a microscope coupled with a Fourier transform (FT) microspectrophotometer, as well as an image analyzer which allowed the images to be processed and stored on a hard disk. The absorption spectra were recorded on a 7.5 µm spot using a 40 x objective. The parameters for recording the spectra were optimized as described by the manufacturer. A halogen lamp was used for bright field observations and measurement of the absorption spectra.

Exposure time of the detector (1024 bits silicon photodiode array detector) to a fringe produced by the interferometer unit (Birefringent Interferometer Monochromator using a Wollaston Prism) was set to 4 s, yielding the corresponding FT spectrum.

*Stentor coeruleus* cells contracted upon exposure to light and remained stationary. To obtain a high S/N ratio it was necessary to use a high light intensity to record the spectra. Under these conditions *Stentor* died after 10 to 15 min. Hence each measurement was performed immediately after preparation of a fresh sample. Absorption and fluorescence emission spectra performed on a 1 or 3 µm sampling spot (using a 100 x objective) were very noisy and of poor resolution. Since the red-light absorbing components have been localized within the pigment granules (Song et al. 1980a, b), a 7.5 µm sampling spot encompassing several pigment granules was selected for this study. This resulted in a higher quality *in situ* absorption spectrum, as shown in Fig. 6. Two major peaks are present with maxima at 609 ± 2 and 619 ± 2 nm. The peak positions correspond with the reported maxima for stentorin I and stentorin II respectively (Kim et al. 1990).

Measurements at different positions on the same cell (Fig. 7) indicate a quite homogenous distribution of pigment granules over the cell body with a higher concentration around the oral pouch of the cell. The peak positions, as determined by the 4th derivative spectroscopy, are indicated on the spectra. The main peak could each time be resolved in two components with maxima surrounding the reported maxima for isolated stentorin I and stentorin II. This observation contrasts with the PAS data in that the latter suggested different localizations for stentorin I and II, *vide supra*. One plausible explanation for the apparent contradiction is that spatial resolution of the microspectrometer used was too broad (7.5 µm) to preferentially excite the pigment granule-bound stentorin II.

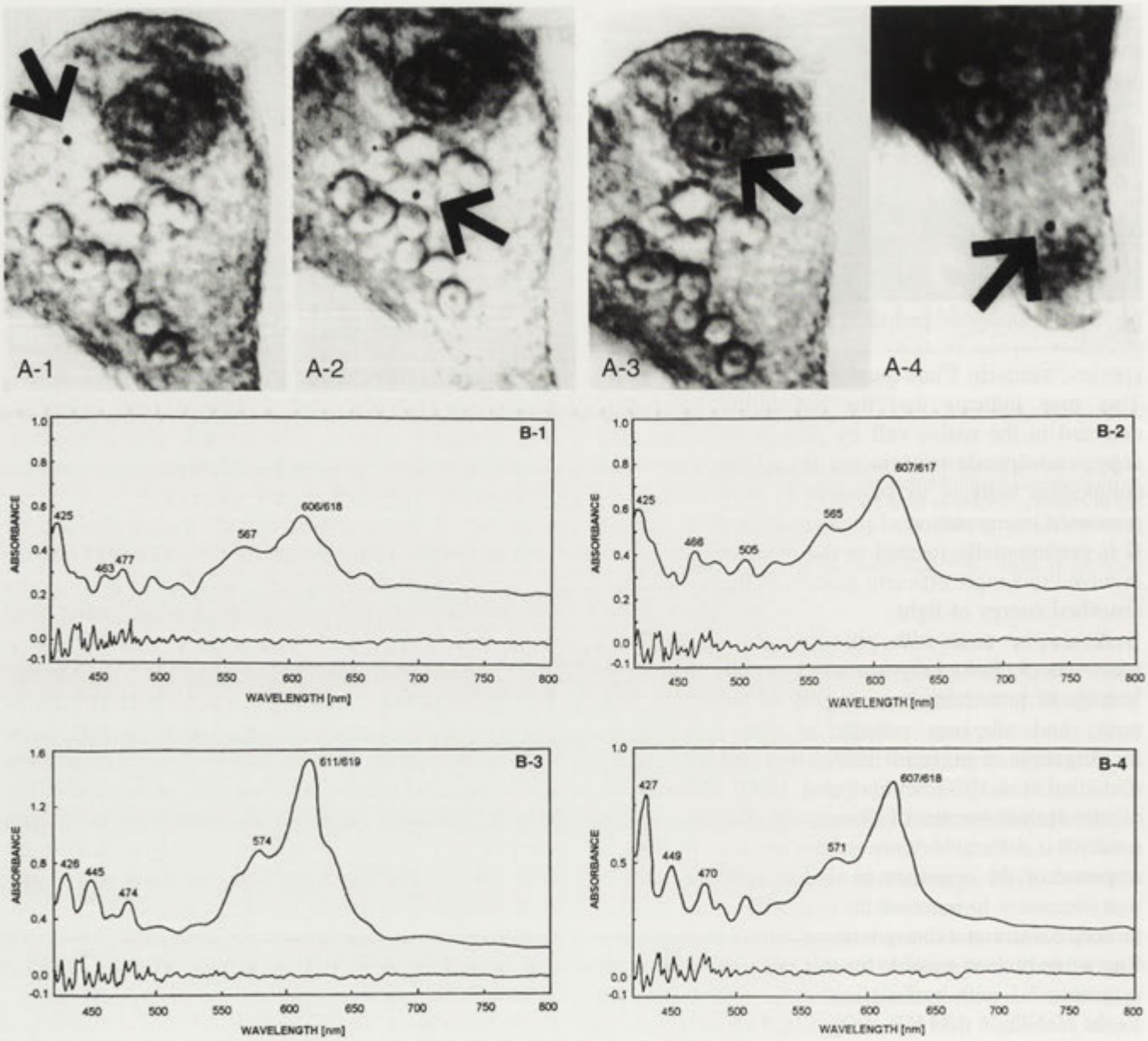



Fig. 7. (A) Transmission photographs of one single *Stentor coeruleus* indicating different sampling spots of the respective absorption spectra (B). The peak positions determined by taking the 4th derivative are indicated on the spectrum (1 and 2 - central parts of the cell, 3 - oral part, 4 - tail part)

The pigment granule in *Blepharisma* has been studied since the 1950s (Inaba et al. 1958, Kennedy 1965). The pigment granules are 0.5 μm in diameter and lying just below the pellicle (Giese 1973). Two types of granules (one dense, homogeneous and spherical; the other less dense, granular and irregular in outline) were observed by Giese (Giese 1973) and the earlier workers (Inaba et al. 1958, Kennedy 1965). A recent study by Matsuoka and others (Matsuoka 1994, personal communication to Song) showed that the granules surrounded by membrane were connected to a plasma membrane. The authors also showed that the pigment granules contain a honeycomb-like structure and a stacked lamella structure. No photoacoustic or microspectrophotometric data are available to describe the localization and the spectroscopy of blepharismin.

## FUNCTION

The exact photochemistry of stentorin is unknown. Preliminary experimental results indicated there is no spectroscopically identifiable photochemical cycle, as

observed in rhodopsin and phytochrome (Dai 1994). Stentorin, although analogous to hypericin which is an efficient photodynamic agent with a high singlet oxygen yield, was demonstrated to be a poor singlet oxygen generator under physiological conditions (Dai et al. 1992). Thus the reactive molecular oxygen might not play a significant role in the light signal transduction in *Stentor coeruleus*.

Stentorin contains several hydroxyl groups. Their $pK_a$ values are likely to decrease upon light excitation (Walker et al. 1979). It was recently reported that 2- or 2'-hydroxyl is the preferred site of deprotonation of hypericin and the $pK_a$ value decreased by 2 units upon light excitation (Falk et al. 1992). Similar deprotonation of stentorin may serve as the initial photosensory transduction step, as suggested earlier (Song 1981). The proton dissociation and transduction during the excited state could also be expected to be facilitated via an acid-base network of amino acid residues of the apoprotein, analogous to the charge relay network of serine proteases (Blow and Steitz 1970). In fact, the photoresponses of *Stentor coeruleus* were shown to be dependent on pH (Walker et al. 1979, Fabczak H. et al. 1993a) and a light-induced pH decrease was observed in *Stentor coeruleus* (Walker et al. 1981). Additionally, intracellular pH-modulating reagents [including ammonium chloride, protonophores carbonylcyanide *m-chlorophenyl-hydrazone (CCCP) and carbonylcyanide p*-(trifluoromethoxy)-phenyl-hydrazone (FCCP)] were found to reduce the photoresposiveness (Fabczak H. et al. 1993a).

A recent pump-probe experiment showed that stentorin II undergoes a rapid absorbance decrease (bleaching) in less than 3 ps; whereas, stentorin free chromophore and the model compound hypericin do not exhibit the ultrafast photoprocess (Savikhin et al. 1993). This process is faster than the fluorescence decay occurring with a lifetime of about 10 ps (Song et al. 1990). The ultrafast bleaching process, monitored at 565-630 nm, may be an intermolecular proton transfer to an appropriately situated amino acid residue in the photoreceptor molecule.

Electron transfer may also be involved in the primary photoprocess of stentorin. It was shown that hypericin photoreduced ferric ions to ferrous ions in DMSO (Song et al. 1991). On the contrary, hypericin and stentorin were found unreactive in the presence of electron donor, such as dithionite (Kim et al. 1990). Recently the formal potentials for hypericin were measured using cyclic voltammetry (Redepenning and Tao 1993). Two reversible one-electron reductions were observed at -0.87 V and -1.18V. Oxidation is observed at +0.90 V. These formal potentials are consistent with the absorption maximum at 599 nm (2.1 eV). Thus, hypericin is not only a good electron acceptor, but also a strong electron donor. As an analog to hypericin, stentorin is expected to be electrochemically similar. However, whether electron transfer plays a primary role in the sensory transduction is still unknown.

Since the photoreceptor molecule in *Blepharisma japonicum* has not been purified, and its structure has not been fully elucidated the photochemistry of blepharismin is even more difficult to describe. Since blepharismin is structurally similar to hypericin and stentorin, we expect that similar photochemistry occurs in blepharismin. An added proof is that modulation of the photophobic response of *Blepharisma japonicum* by ammonium chloride, CCCP, and FCCP were observed, as in *Stentor coeruleus* (Fabczak H. et al. 1993a). A pH decrease, upon light irradiation, was observed in *Blepharisma japonicum* (Matsuoka et al. 1992b).

Recent investigation of the bioelectrical response in *Stentor coeruleus* and *Blepharisma japonicum*, induced by light stimulation, indicated that the membrane potential change is correlated with behavior response (Fabczak S. et al. 1993a, b). The light-induced membrane depolarization occurred with a latency of about 0.5 s, suggesting a biochemical process preceding the membrane depolarization. It was shown that light-induced cell elongation in *Blepharisma japonicum* was mediated by changes in cyclic nucleotide concentration (Ishida et al. 1989). 8-Bromo-guanosine 3', 5'-cyclic monophosphate (a membrane permeable analog of guanosine 3', 5'-cyclic monophosphate (cGMP)), pertussis toxin (a G-protein activity modulator) and 3'-isobutyl-methylxanthine (an inhibitor of cGMP phosphodiesterase), exhibited similar effects in reducing the photoresponsiveness in *Stentor coeruleus* (Fabczak H. et al. 1993b, c). It appears that cGMP is a possible signal transmitter in the photosensory signal cascade in *Stentor coeruleus* and *Blepharisma japonicum*. A transducin-like G-protein was detected using the α-transducin antibody (Fabczak H. et al. 1993b). However, a complete understanding of how these transducers and secondary messengers couple to the photoreceptors, and the ciliary reversal awaits the characterization of the native photoreceptors.

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# Proteolytic Activity in Extracts of *Entamoeba invadens* and *E. histolytica*: A Comparative Study

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**Summary.** Two species of amoebae: *Entamoeba invadens* and *Entamoeba histolytica* are potentially invasive in reptiles and certain primates, respectively. Since proteinases may be involved in pathogenic mechanisms (tissue necrosis), at least in *E. histolytica*, it was of interest to compare their proteases. We compared the sensitivity of proteolytic activity in extracts of both species of amoebae to pH, temperature, and proteinase inhibitors of different catalytic classes. Proteinases in extracts of *E. invadens* were mainly inhibited in the presence of chelating agents, in contrast to the partial or complete activation for proteinases of *E. histolytica*. The pH-dependence curves for both types of extracts were also different. When hide powder azure was used as a substrate, extracts of *E. histolytica* showed a maximum of activity at pH 7 and a shoulder at pH 5, while extracts of *E. invadens* only showed a maximum at pH 7.4. With azocasein as substrate, extracts of *E. invadens* had two maxima of activity at pH 6 and 7.4, while extracts of *E. histolytica* had a maximum at pH 6 and a shoulder at pH 7.4. The temperature optimum for the digestion of azocasein was 60°C for extracts of *E. invadens* and *E. histolytica*, but proteolytic activity in the latter was more resistant to temperature.

Analysis of lysates from both species of amoebae by substrate-gel electrophoresis, also showed marked differences in the observed lysis zones. *E. histolytica* showed six hydrolysis zones at with relative molecular masses of 66, 58, 42, 31, 28, and 23 kDa; and *E. invadens* showed only two zones, with relative molecular masses of 52 and 45 kDa. We conclude that although both species of amoebae contain mainly cysteine proteinases, the enzymes contained in both types of cells are different.

**Key words.** *Entamoeba invadens, E. histolytica*, proteolytic activity, inhibitor, substrate gel electrophoresis.

## INTRODUCTION

*Entamoeba invadens* is a protozoan that can produce amebiasis in carnivorous reptiles (Meerovitch and Chadee 1988). Although the precise mechanisms by which this parasite invades its host are unknown, it is possible that tissue necrosis in the affected reptiles is partly caused by proteases of the parasites, as has been postulated recently for *E. histolytica*, using a model of acute experimental amebiasis in the rat (Becker et al. 1988).

There are numerous studies about cysteine proteinases of *E. histolytica* (Lushbaugh 1988). Several distinct proteinases have been purified and three genes have been sequenced and characterized (Keene et al. 1986, Luaces and Barrett 1988, Lushbaugh et al. 1985,

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Scholze and Werries 1984, Reed et al. 1993, Tannich et al. 1992). The sequences indicate that *E. histolytica* has two cysteine proteinases belonging to the papain superfamily and one with homology to cathepsin L (Reed et al. 1993).

Scholze and Schulte (1990) purified a 28 kDa cysteine proteinase form *E. invadens* that had similar properties to one of the cysteine proteinases from the papain superfamily of *E. histolytica*, but it also had some differences, e.g., the temperature optimum and the immunological reactivity. Apparently these enzymes from two different species of *Entamoeba* have no epitopes in common.

A different type of information can be obtained if proteolytic activity is studied in more complex mixtures like extracts or lysates. The enzymes of different catalytic classes contained in these preparations probably act simultaneously when amoebae are lysed inside the host producing numerous and complex effects. Besides, the predominant catalytic class and other parameters like pH and temperature optima, substrate specificity, etc., for the mixture, can only be known by studying the properties of these complex systems.

In a previous study Avila et al. (1985) found that the main catalytic class of proteolytic enzymes in extracts of *E. invadens* and *E. histolytica* is that of cysteine. Using inhibitors of the four catalytic classes of proteinases, we confirmed and extended their findings for *E. histolytica* lysates (Perez-Montfort et al. 1987). The purpose of this study was to compare the proteolytic activity in extracts of *E. invadens* and *E. histolytica* to find out their similarities and differences and gain information about the proteinases produced by these two amoebae.

## MATERIALS AND METHODS

*Entamoeba invadens*, strain PZ, and *E. histolytica*, strain HM 1: IMSS, were cultured axenically in BI-S-33 medium at room temperature and 36.5°C, respectively (Diamond 1983). Cells were harvested at the end of the logarithmic growth phase for all experiments. Amebic extracts using Triton X-100 and amebic lysates were obtained by procedures described previously (Perez-Montfort et al. 1987). Protein was determined by the Biuret reaction.

Proteolytic activity of the extracts was determined in buffers with different pH values using either azocasein, azocoll, hide powder azure as the substrate, also by described procedures (Avila et al. 1985, Perez-Montfort et al. 1987).

The inhibitors used were antipain, aprotinin, ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis(β-amino-ethylether) N, N'-tetraacetic acid (EGTA), iodoacetamide (IA), leupeptin, N-ethylmaleimide (NEM), pepstatin A, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), from Sigma Chemical Co., St. Louis, MO, (N-[N-(L-3 trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) (E-64), [N- (α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan] (phosphoramidon), from Boehringer, Mannheim, and human α-2-macroglobulin (α2m), purified according to the method of Kurecki et al. (1979).

Digestion of gelatin on X-ray films was adapted from the method of Cheung et al. (1991) as follows: lysates from a known number of trophozoites were prepared in Tris-HCl 0.05 M pH 7.5 and adjusted to final volume of 50 µl with the same buffer. These samples were applied onto X-Omat XAR-5 film that had been previously exposed, developed, fixed, washed and dried. The film was incubated at 42°C for 96 h. The film was washed with tap water at room temperature from a laboratory faucet and digestion was apparent as clear zones in the film.

Substrate-gel electrophoresis was performed in separating gels containing 12.5% polyacrylamide and using gelatin or casein as substrates, as previously described (Ostoa-Saloma et al. 1989, Perez-Montfort et al. 1987).

## RESULTS

The proteolytic activity in *Entamoeba invadens* and *E. histolytica* extracts varied in different experiments over a three-fold range. Extracts of *E. invadens* showed consistently more activity (sometimes up to five-times more), in our assay with azocasein, than extracts containing the same amount of protein or of an equivalent number of trophozoites of *E. histolytica*. Some representative results are shown in Table 1. When hide powder azure or azocoll were used as substrates, we observed approximately the same activity per number of amoebae with both types of extracts.

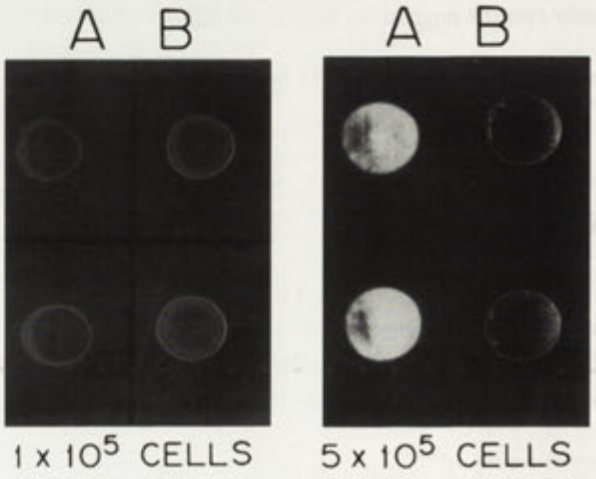


Fig. 1. Digestion of the gelatin coating on X-ray film with lysates of trophozoites in duplicate. A - *E. invadens* and B - *E. histolytica*

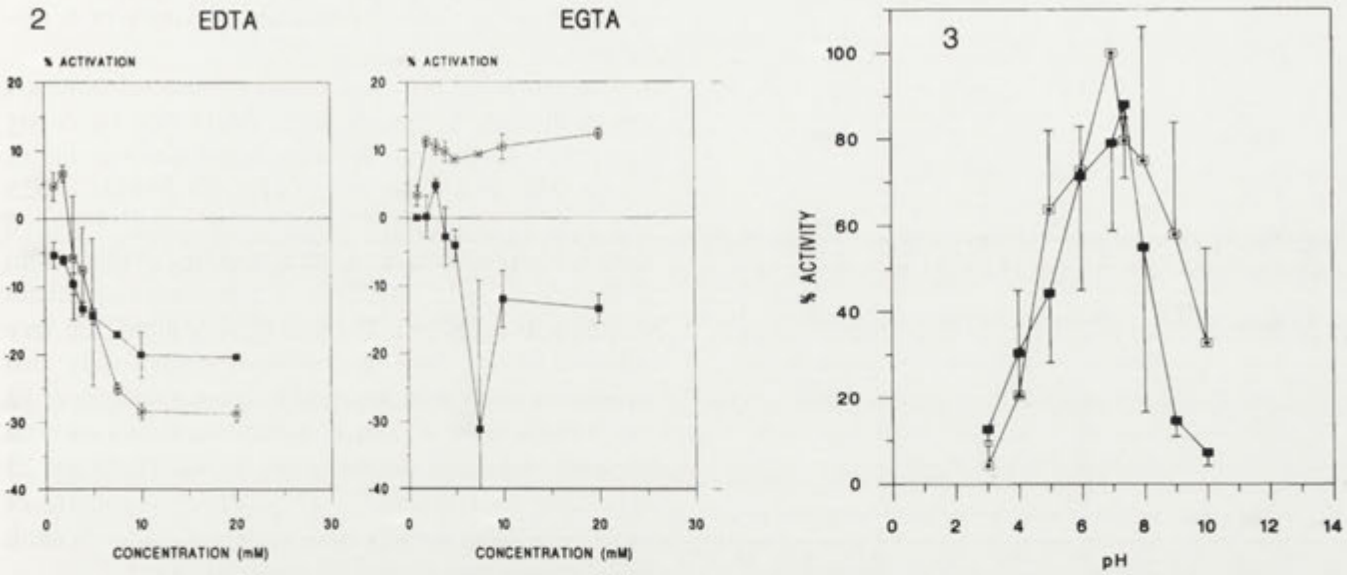


Fig. 2. Effect of EDTA and EGTA on the proteolytic activity of extracts of *E. invadens* (filled symbols) and *E. histolytica* (empty symbols) on azocasein. The bars represent standard deviation of triplicate determinations

Fig. 3. Comparison of the pH-dependence of proteolytic activity in extracts of *E. invadens* (filled symbols) and *E. histolytica* (empty symbols) on hide powder azure. The bars represent standard deviation of triplicate determinations

Lysates of *E. invadens* also showed more activity than those of *E. histolytica*, when the method to detect gelatin digestion on X-ray films was used (Fig. 1). The difference is more evident with lysates corresponding to $5 \times 10^5$ cells.

The effect of the different inhibitors used on the proteolytic activity of extracts from both species of amoebae can be seen in Table 2. The inhibitors of cysteine proteinases, IA, PHMB, NEM, and E-64 reduced the activity in both cases by 80 to 103%. The inhibitors that inhibit both serine and cysteine proteinases, antipain, leupeptin, TLCK, TPCK, and a combination of PHMB and SBTI, also reduced the activity by 70 to 109%. These results indicate that the proteolytic activity in extracts of *E. invadens* and *E. histolytica* is mainly due to cysteine proteinases. Inhibitors of serine proteinases such as aprotinin, PMSF and SBTI can inhibit anywhere from 0 to 20 % of the activity in both types of extracts, suggesting that a small proportion of the total proteolytic activity may be due to proteases with that catalytic mechanism. Neither pepstatin A nor phosphoramidon had any effect on the activity of both types of extracts, indicating that proteases belonging to the catalytic classes of the aspartic and metalloproteinases, respectively, account for an insignificant amount of the proteolytic effect observed.

The chelating agents EDTA and EGTA, which are used for inhibition of metalloproteinases at concentrations of 1 or 2 mM, had different effects on the activity of both types of extracts.

Extracts of *E. invadens* were inhibited by EDTA at all concentrations tested while extracts of *E. histolytica* had a small activating effect at very low concentrations and an inhibiting effect at higher concentrations, reaching a plateau of approximately 30% inhibition at 10 mM (Fig. 2). For extracts of *E. invadens* EGTA had a small activating effect at low concentrations ( <4 mM) and an inhibitory effect at higher concentrations ( >5 mM), but extracts of *E. histolytica* were activated at all concentrations tested (Fig. 2)(see also Fig. 1 in Perez-Montfort et al. 1987).

In a pH-dependence curve using hide powder azure as substrate, extracts of *E. invadens* show a maximum activity at pH 7.4 (Fig. 3). The pH-dependence curve of extracts of *E. histolytica* show maximum activity at pH 7 and a shoulder at pH 5, reproducing previously published results (Avila et al. 1985). The variation for the results with this substrate is so great that there is no statistical difference between the curves. In contrast, the profile observed for both species of amoeba is different when azocasein is used as a substrate (Fig. 4). Extracts of *E. invadens* showed two maxima, at pH 6 and 7.4, but extracts of *E. histolytica* showed maximum activity at pH 6 and a shoulder at pH 7 and 7.4. A comparison of temperature-dependence of proteolytic activity of both types of extracts on azocasein, showed that they

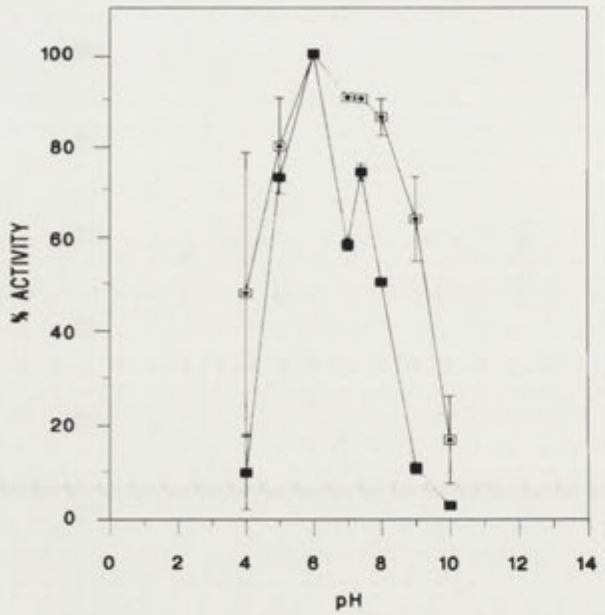


Fig. 4. Comparison of the pH-dependence of proteolytic activity in extracts of *E. invadens* (filled symbols) and *E. histolytica* (empty symbols) on azocasein. The bars represent standard deviation of triplicate determinations

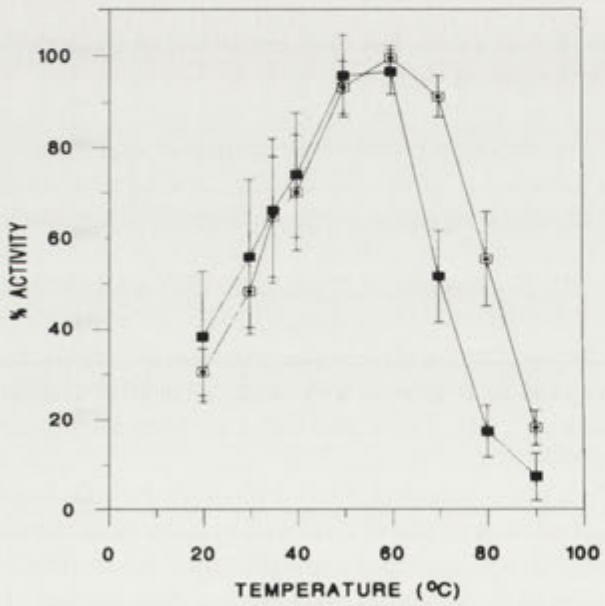


Fig. 5. Comparison of temperature-dependence of proteolytic activity in extracts of *E. invadens* (filled symbols) and *E. histolytica* (empty symbols) on azocasein. Assays were made at pH values of 7.5 for 30 min. The bars represent standard deviation of triplicate determinations

had a maximum activity at 60°C (Fig. 5). Extracts of *E. invadens* had 49% of maximum activity at 70°C while extracts of *E. histolytica* still had approximately 90 %. At 80°C and 90°C the values were 17% and 8% for *E. invadens* and 57% and 18% for *E. histolytica*, respectively; indicating greater stability at high temperatures of the proteolytic activity contained in extracts of *E. histolytica*.

Our group has previously observed that substrate gel electrophoresis of lysates of *E. histolytica* yields six hydrolysis zones with apparent molecular weights of 80-60 kDa, 56-45 kDa, 40-35 kDa, 33-29 kDa, 30-25 kDa, and 23 kDa (Ostoa-Saloma et al. 1989), Figure 6 shows a representative result comparing lysates from *E. histolytica* and *E. invadens* on gels containing gelatin or casein as substrates. The hydrolysis patterns are very different. On gelatin *E. invadens* shows only two hydrolysis zones with apparent molecular weights of 52 and 45 kDa (lane A) and *E. histolytica* shows six with apparent molecular weights of 66, 58, 42, 31, 28 and 23 kDa (lane B), respectively. These different patterns of hydrolysis zones are repeated on a gel containing casein as substrate (lanes C and D, respectively).

## DISCUSSION

The results of this work confirm those previously reported by Avila et al. (1985) in which they show that the main catalytic class in extracts of *E. invadens* is that of cysteine proteases, and also extend their findings showing that most inhibitors of all catalytic classes of proteases act in a similar way on extracts from both species of amoebae (Table 2).

Extracts of trophozoites of *E. invadens* also show great and random variability of proteolytic activity, as was previously reported for extracts of *E. histolytica* (Perez-Montfort et al. 1987). These results are also in accordance with the variability shown by *E. invadens* to digest whole erythrocytes (Ramírez-Córdova et al. 1990). The reason for this variability is unknown.

It is also interesting that extracts of *E. invadens* show a higher proteolytic activity per amoeba, using azocasein or gelatin as substrates, when compared with extracts of *E. histolytica* (Table 1, Fig. 1). This is not the case when hide powder azure or azocoll are used. These results suggest that *E. invadens* contains one or a group of proteinases which are more active at degrading azocasein or gelatin. We speculate that this might be related to the different histolytic capacity shown by both species of amoebae on reptilian and mammalian tissues.

The differences observed between the inhibition of proteolytic activity in extracts of *E. invadens*, with EDTA and EGTA (Fig. 2), also suggest that there might be qualitative differences in the proteases produced by both types of organisms. Further support came from a

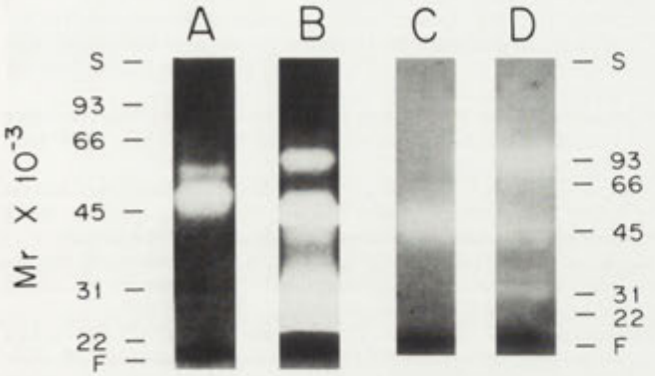


Fig. 6. Zymogram of gels on which lysates of 2 X $10^4$ amoebae were analyzed. A and C - *E. invadens* and B and D - *E. histolytica*. One substrate gel contained gelatin (lanes A and B) the other substrate gel contained casein (lanes C and D). S marks the end of the stacking gel, F is the dye front. The scale for $M_r$ on the left is for lanes A and B and the one on the right is for lanes C and D

Table 1

Comparison of the relative proteolytic activity on different substrates by extracts of *E. invadens* and *E. histolytica*

| Substrate | *E. invadens* (% Activity) | | *E. histolytica* (% Activity ± S.D.) | |
|---|---|---|---|---|
| | 4 X $10^4$ cells | 777 µg | 4 X $10^4$ cells | 777 µg |
| Azocasein | 100 | 100 | 60 ± 8 | 38 ± 5 |
| Azocoll | 100 | 100 | 96 ± 12 | 78 ± 6 |
| Hide Powder Azure | 100 | 100 | 94 ± 3 | 83 ± 8 |

Table 2

Comparison of the effect of different inhibitors on proteolytic activity of amebic extracts on azocasein

| Inhibitor(s) | Class ...* | % Inhibition ± S.D. | | |
|---|---|---|---|---|
| | | *E. invadens* | *E. histolytica* | |
| IA (2 mM) | c | 103 ± 2 | 92 ± 5 | † |
| PHMB (1 mM) | c | 96 ± 1 | 92 ± 2 | † |
| NEM (2 mM) | c | 86 ± 9 | 84 ± 2 | † |
| E-64 (140 µg/ml) | c | 96 | 90 | |
| PMSF (2 mM) | s | 9 ± 8 | 15 ± 5 | † |
| SBTI (100 µg/ml) | s | 16 ± 3 | 18 ± 9 | † |
| Aprotinin (100 µg/ml) | s | 20 ± 2 | 12 ± 4 | |
| Pepstatin A (1 µg/ml) | a | -6‡ ± 9 | 0 | † |
| Phosphoramidon (100 µg/ml) | m | 2 ± 1 | 0 ± 2 | |
| EDTA (2 mM) | m | 8 ± 4 | -13 ± 1 | † |
| EDTA (20 mM) | m | 31 ± 6 | 33 ± 13 | † |
| EGTA (2 mM) | m | -4 ± 3 | -17 ± 4 | † |
| EGTA (20 mM) | m | 5 ± 2 | -22 ± 2 | † |
| Antipain (50 µg/ml) | c + s | 80 ± 0 | 75 ± 1 | |
| Leupeptin (50 µM) | c + s | 71 ± 11 | 79 ± 1 | † |
| TLCK (50 µg/ml) | c + s | 97 ± 0 | 95 ± 1 | |
| TPCK (150 µg/ml) | c + s | 93 ± 3 | 91 ± 0 | |
| PHMB (1 mM) + SBTI (100 µg/ml) | c + s | 93 ± 3 | 109 ± 9 | † |
| α2m (5 mg/ml) | all | 98 ± 2 | 77 ± 8 | † |

* Class of endopeptidase inhibited: c - cysteine, s - serine, a - aspartic, m - metalloproteinases
† - data for *E. histolytica* from Perez-Montfort et al., 1987.
‡ - activation

similar and a different pH curve observed with lysates of both types of organisms using two different substrates (Figs. 3, 4), from the different temperature-dependence curves (Fig. 5), and from substrate gel electrophoresis, in which the hydrolysis pattern is very different (Fig. 6). Thus, four types of evidence indicate that *E. invadens* has qualitatively different proteinase molecules than *E. histolytica*. These results are in contrast to those obtained for the only cysteine proteinase purified from *E. invadens* (Scholze and Schulte 1990) in which many of the characteristics of this enzyme were very similar to those of a purified cysteine proteinase of *E. histolytica*. Since in extracts one measures the interaction of many activities, our result would predict that other proteolytic enzymes in the two types of extract should have different properties.

The pH-dependence curve for extracts of *E. histolytica*, with hide powder azure as substrate (Fig. 3), essentially reproduced the results of Avila et al. (1985). The pH-dependence curve with azocasein was different, but, differences in the pH optima for the hydrolysis of proteins with the same enzyme have been frequently observed (North 1982).

The temperature optimum for both types of extracts was 60°C. The stability of proteolytic activity in *E. histolytica* extracts at high temperatures, may in part be explained by the temperature optimum of 85°C, reported by Scholze and Werries (1984), for the 21 kDa proteinase partially purified by them. We think this enzyme is present in our extracts and corresponds to one of the three low molecular weight hydrolysis zones seen in the zymograms (Fig. 6, lane B). On the other hand, the proteases in extracts of *E. invadens* seem to denature rapidly at temperatures above 60°C and the low molecular weight proteinase, with a temperature optimum of 48°C, purified by Scholze and Schulte (1990), is not apparent as a distinct hydrolysis zone in the zymograms.

These differences in heat stability of the proteolytic enzymes may be related to the fact that these parasites have adapted to growth in different temperature environments. The temperatures at which *E. invadens* and *E. histolytica* are grown are 25°C and 37°C, respective-

ly, and both of these strains have been in culture for extended periods of time.

It has been previously noted that, quantitatively, the most important group of proteolytic enzymes in protozoa is that of cysteine proteinases (North et al. 1990). *E. histolytica* and *E. invadens* are no exception to this observation. It has also been observed that these cysteine proteinases often have a molecular weight of about 25 kDa and have other properties that are consistent with their belonging to the papain superfamily (Barrett 1986, North et al. 1990). *E. histolytica* has at least two (Reed et al. 1993 and Fig. 6) proteinases that fulfill this requirement, but, interestingly, zymograms of *E. invadens* lysates did not show any lysis zones in that molecular weight range. It is possible that in *E. invadens* such an enzyme is in minimal quantities, that the conditions in which the zymograms were made are inhibitory to its activity, or that it forms very stable polymers.

Further work along these lines, including sequencing and comparison of cysteine proteases obtained from both types of organism, but particularly from *E. invadens*, could prove useful in clarifying this point.

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# Effects of Copper on Ciliate Communities from Activated Sludge Plants

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**Summary.** The toxic effects of copper were studied in ciliate communities, taken from activated sludge of sewage treatment plants. Experiments were performed with and without oxygen addition using copper concentrations ranging from 1 to 10 ppm and the results were compared to a control. The toxic effects of copper in the studied communities can be assessed either by means of total number of ciliates or specific diversity. Thus both parameters were decreased by the toxic action of Cu. Detailed observation of copper-induced reduction of organism concentration showed that not all species were reduced in the same way producing changes in the dynamics of ciliate communities that masked precise calculation of median tolerance limit concentrations. These variations of toxic effects of copper depended on the sewage treatment plant which the sludge sample had been taken from, observing an inverse relation between toxicity and sludge age. All this suggests that in addition to time of exposure to the toxic, other factors as dissolved oxygen, and sludge age should be taken into account in toxicity tests.

**Key words.** Toxicity, Cu, protozoa, Ciliophora, activated sludge.

## INTRODUCTION

In biological waste-water treatment plants heavy metals conveyed by the waste water are mostly retained in the biofloc. In a comparative study of biological systems of sewage treatment, Hannah et al. (1986) found that the activated sludge system is the most effective way to eliminate heavy metals, removing 82% of the copper from an inflow containing 0.280 ppm. Barth et al. (1965) however, reported that in order to avoid deterioration of the treatment the concentration of Cu in the inflowing sewage must be below 1 ppm in continuous dosage or below 75 ppm for a maximum of four hours, and noted that the unretained metal would be dissolved in the effluent water. This percentage of Cu, which is difficult to measure, may modify the structure and dynamics of ciliate populations.

Sudo and Aiba (1973) found that a concentration of between 0.25 and 0.27 ppm of copper halves the specific growth rate of *Vorticella microstoma* and *Opercularia* sp. from activated sludge.

Toxicology studies on *Tetrahymena pyriformis* have provided valuable information on the effects of copper on ciliate protozoa such as complete inhibition of growth at 1 mM Cu (Yamaguchi et al. 1973), reduction of copper toxicity by accumulation of Cu in small refractile intracellular granules (Nilsson 1981) and inhibition of respiration at 0.1 mM Cu (Wakatsuki et al. 1986).

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Although many studies of Cu toxicity in ciliates have been carried out in monoaxenic cultures, few toxicology studies have been reported on ciliate communities. Ruthven and Cairns (1973) used the percentage of surviving species to assess the effect of copper on protozoan communities, which can be a rough method when work is done in communities with a low number of species. Dive and Persoone (1984) however, stated that the use of protozoan diversity index in ecotoxicological studies is a simplification that might be interesting when many samples are analyzed. Copper may also have an effect on the trophic relations of protozoan communities which was studied by Doucet and Maly (1990) reporting disruption of the predator-prey relationship between *Didinium* and *Paramecium*.

The aim of this study was to provide data that may contribute to our knowledge on ecotoxicology and in the control of activated-sludge waste-water treatment plants. Using laboratory tests we studied the toxic effects of copper on communities of ciliate protozoa from activated-sludge together with specific interactions and variations in the diversity index.

## MATERIALS AND METHODS

Samples for bioassay were taken from three activated-sludge waste-water treatment plants. The following parameters were determined: sludge age, pH, dissolved oxygen, volatile suspended solids from aeration tank (MLSS, MLVSS), and $BOD_5$. Copper concentration in aeration tank was measured by induction-coupled plasma. Physical and chemical factors were measured using the techniques recommended by American Public Health Association (Standard Methods 1989). Sludge age was measured according to Metcalf-Eddy (1979).

Material for study was placed in 250 ml Erlenmeyer flasks. Each flask held 20 ml of sludge, 160 ml of water from the secondary settling tank and 20 ml of copper sulphate at various concentrations. Final copper concentration in the flasks were 0 (control), 1, 2.5, 5 and 10 ppm. In some experiments oxygen was supplied with a controlled compressor, maintaining oxygen at 4.8-6.6. ppm while other experiences were carried out without oxygen addition. Analyses were performed at 20°C and pH 6.5-7.5. To have more feasible results all experiments were carried out in duplicates.

In the article the different experiments are coded as follows:

Experiment 1n: sludge taken from plant 1, no oxygen is added.

Experiment 2n: sludge taken from plant 2, no oxygen is added.

Experiment 2a: sludge taken from plant 2, oxygen is added.

Experiment 3n: sludge taken from plant 3, no oxygen is added.

Experiment 3a: sludge taken from plant 3, oxygen is added.

Protozoan quantification was made following Salvadó and Gracia (1993), thus four subsamples (50µl each) of each bioassay flask were extracted with an automatic micropipette, and the number of organisms of each taxon were counted under an optical microscope: magnification x100 for ciliates and magnification x400 for flagellates less than 20µm and gymnamoebia of less than 50µm.

Ciliates were identified following Kahl (1930-1935), Curds (1982), Curds et al. (1983), Foissner and Schiffmann (1974) and Lee et al (1985), using special staining techniques such as ammoniacal silver carbonate (Fernandez-Galiano 1976) or silver nitrate (Klein 1926).

Specific diversity of ciliates was calculated according to Shannon-Weaver (1957).

Results are expressed in bits/individual. In the cases in which no ciliates survived diversity was considered to be 0.

The median tolerance limits (TLm) or concentration for a 50% survival (Doudoroff et al. 1951) were calculated for the species of ciliates present in at least two of the plants the sludges were taken from.

## RESULTS

### Physical and chemical parameters in the sewage treatment plants

The values of physical and chemical parameters of the sewage treatment plants at the moment of sample extraction show that they operated in different conditions (Table 1). It has to be noted that the highest sludge age was found in plant 3, followed by plant 2 and plant 1. Analysis of soluble Cu in the samples of activated sludge from the three plants gave very low concentrations ranging from 0.01 to 0.05 ppm.

### Effects of copper on the concentration of protozoa and on specific diversity of ciliates

The concentration of the different species of ciliates and other protozoa as well as the specific diversity along each bioassay are found in Tables 2, 3 and 4. The evolution (with respect to control assays at 0h) of diversity together with the concentration of ciliates along the

Table 1

Physical and chemical parameters of the waste-water treatment plants

| | Plant 1 | Plant 2 | Plant 3 |
|---|---|---|---|
| Sludge age, days | 4.3 | 7 | 10 |
| BOD5 Primmary settling tank, ppm | 270 | 220 | 170 |
| BOD5 Secondary settling tank, ppm | 26 | 25 | 7 |
| MLSS, ppm | 1800 | 1900 | 2000 |
| MLVSS, ppm | 1620 | 1800 | 1400 |
| Daily average of D.O. in the final track of aeration tank, ppm | 2.4 | 1.2 | 3.7 |

Table 2

Experiment 1n (Sludge taken from plant 1, no oxygen addition): Effects of different concentrations of Copper on the concentration of the different species of ciliates and other protozoa as well as on diversity in bioassay

| | Time | Contr. | ppm of Cu 1 | 2.5 | 5 | 10 |
|---|---|---|---|---|---|---|
| Organisms in ind/ml | | | | | | |
| *Opercularia* | | | | | | |
| *coarctata* | 0h | 278 | | | | |
| | 24h | 223 | 207 | 82 | 6 | 0 |
| | 48h | 190 | 88 | 69 | 9 | 0 |
| *Aspidisca* | | | | | | |
| *cicada* | 0h | 299 | | | | |
| | 24h | 639 | 0 | 0 | 0 | 0 |
| | 48h | 494 | 0 | 0 | 0 | 0 |
| *Vorticella* | | | | | | |
| *microstoma* | 0h | 297 | | | | |
| | 24h | 224 | 264 | 156 | 0 | 0 |
| | 48h | 304 | 38 | 25 | 0 | 0 |
| *Acineria* | | | | | | |
| *uncinata* | 0h | 85 | | | | |
| | 24h | 680 | 138 | 16 | 0 | 0 |
| | 48h | 1748 | 164 | 4 | 0 | 0 |
| *Chilodonella* | | | | | | |
| *uncinata* | 0h | 100 | | | | |
| | 24h | 88 | 44 | 16 | 0 | 0 |
| | 48h | 57 | 38 | 0 | 0 | 0 |
| *Uronema* | | | | | | |
| *marinum* | 0h | 4 | | | | |
| | 24h | 27 | 0 | 0 | 0 | 0 |
| | 48h | 95 | 0 | 0 | 0 | 0 |
| CILIATES | | | | | | |
| TOTAL | 0h | 1063 | | | | |
| | 24h | 1881 | 653 | 270 | 6 | 0 |
| | 48h | 2888 | 328 | 98 | 9 | 0 |
| FLAGELLATES | | | | | | |
| (x1000) | 0h | 101 | | | | |
| | 24h | 175 | 8 | <1 | <1 | <1 |
| | 48h | 283 | <1 | <1 | <1 | <1 |
| GYMNANOEBIA | | | | | | |
| (x1000) | 0h | 0.5 | | | | |
| | 24h | <1 | <1 | <1 | <1 | <1 |
| | 48h | <1 | <1 | <1 | <1 | <1 |
| DIVERSITY OF CILIATES, bits/ind | | | | | | |
| | 0h | 2.18 | | | | |
| | 24h | 2.08 | 1.79 | 1.46 | 0.00 | 0.00 |
| | 48h | 1.75 | 1.73 | 1.05 | 0.00 | 0.00 |

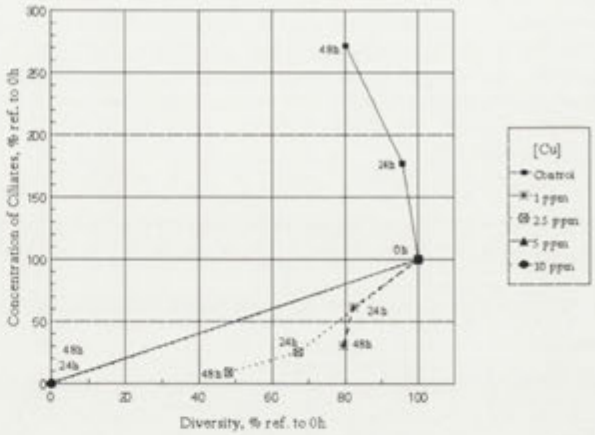


Fig. 1. Evolution of ciliate specific diversity of ciliates together with ciliate concentration in Experiment 1n (sludges taken from plant 1, no oxygen addition). Data are expressed as the percentage of the values for each corresponding parameter in the control bioassay at 0h

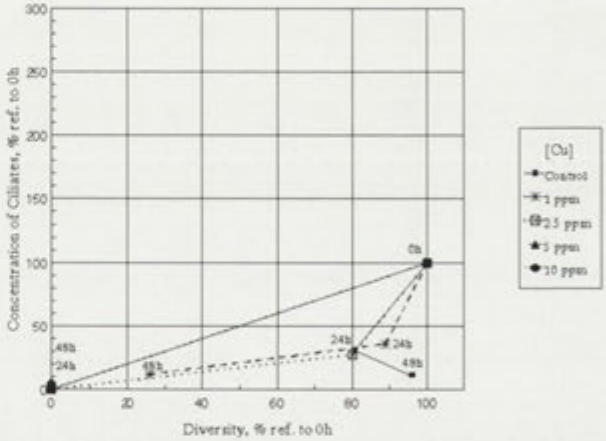


Fig. 2. Evolution of ciliate specific diversity of ciliates together with ciliate concentration in Experiment 2n (sludges taken from plant 2, no oxygen addition). Data are expressed as the percentage of the values for each corresponding parameter in the control bioassay at 0h

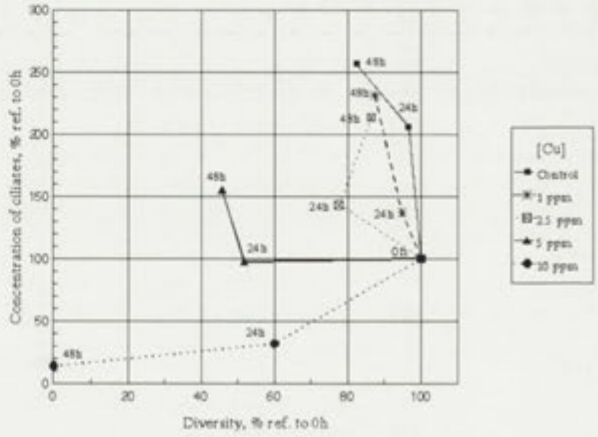


Fig. 3. Evolution of ciliate specific diversity of ciliates together with ciliate concentration in Experiment 2a (sludges taken from plant 2, oxygen was added). Data are expressed as the percentage of the values for each corresponding parameter in the control bioassay at 0h

Table 3

Experiment 2n (Sludge taken from plant 2, no oxygen addition) and Experiment 2a (Sludge taken from plant 2, oxygen was added): Effects of different concentrations of copper on the concentration of the different species of ciliates and other protozoa as well as on diversity in bioassay

| | Time | Contr. | ppm of Cu 1 | 2.5 | 5 | 10 |
|---|---|---|---|---|---|---|
| Experiment 2n, organisms in ind/ml | | | | | | |
| *Vorticella microstoma* | 0h | 116 | | | | |
| | 24h | 75 | 75 | 66 | 10 | 15 |
| | 48h | 14 | 42 | 0 | 0 | 0 |
| *Opercularia minima* | 0h | 170 | | | | |
| | 24h | 19 | 26 | 28 | 0 | 0 |
| | 48h | 13 | 4 | 0 | 0 | 0 |
| *Uronema marinum* | 0h | 99 | | | | |
| | 24h | 25 | 39 | 13 | 0 | 0 |
| | 48h | 18 | 0 | 0 | 0 | 0 |
| CILIATES TOTAL | 0h | 385 | | | | |
| | 24h | 119 | 140 | 107 | 10 | 15 |
| | 48h | 45 | 46 | 0 | 0 | 0 |
| FLAGELLATES (x1000) | 0h | 22 | | | | |
| | 24h | 20 | 10 | 7 | <1 | <1 |
| | 48h | 19 | 9 | <1 | <1 | <1 |
| GYMNANOEBIA (x1000) | 0h | 27 | | | | |
| | 24h | 25 | 16 | 50 | 15 | 11 |
| | 48h | 19 | 9 | <1 | <1 | <1 |
| Experiment 2a, organisms in ind/ml | | | | | | |
| *Vorticella microstoma* | 24h | 294 | 215 | 130 | 100 | 69 |
| | 48h | 400 | 509 | 400 | 129 | 0 |
| *Opercularia minia* | 24h | 215 | 200 | 364 | 280 | 55 |
| | 48h | 509 | 215 | 332 | 475 | 53 |
| *Uronema marinum* | 24h | 294 | 119 | 66 | 0 | 0 |
| | 48h | 91 | 178 | 100 | 0 | 0 |
| CILIATES TOTAL | 24h | 803 | 534 | 560 | 380 | 124 |
| | 48h | 1000 | 902 | 832 | 604 | 53 |
| FLAGELLATES (x1000) | 24h | 99 | 51 | 10 | <1 | <1 |
| | 48h | 85 | 134 | 18 | <1 | <1 |
| GYMNANOEBIA (x1000) | 24h | 55 | 20 | 35 | 95 | 18 |
| | 48h | 49 | 28 | 55 | 152 | 20 |
| DIVERSITY OF CILIATES, bits/ind | | | | | | |
| Experiment 2n | 0h | 1.55 | | | | |
| | 24h | 0.93 | 1.06 | 0.88 | 0.14 | 0.18 |
| | 48h | 0.55 | 0.42 | 0.00 | 0.00 | 0.00 |
| Experiment 2a | 0h | 1.55 | | | | |
| | 24h | 1.57 | 1.54 | 1.26 | 0.83 | 0.99 |
| | 48h | 1.34 | 1.42 | 1.40 | 0.75 | 0.00 |

bioassay of each experiment is shown in Figs. 1, 2, 3, 4 and 5.

As a general trend it can be observed that either the total number or the specific diversity of ciliates were reduced as copper concentration or toxic exposure time increased. However variations in this pattern were frequently observed depending on the origin of the sludges and also on the addition of oxygen.

**Experiment 1n (sludge from plant 1, no oxygen is added):**

In this experiment (Fig. 1) control assays showed a light reduction of diversity together with a strong increase in total number of ciliates along the experiment, which was due mainly to the growth of *Acineria uncinata* and in a lower level *Aspidisca cicada* and *Uronema marinum* (Table 2).

In the intoxicated bioassay two different kinds of responses should be differentiated: at low copper concentrations (1 and 2.5 ppm) a decrease in both diversity and total number of ciliates could be observed, being the decrease in the number of ciliates much clearer. At high concentrations of toxic (5 and 10 ppm) however, diversity and concentration of ciliates decreased fast almost reaching 0 at 24h.

**Experiment 2n (sludge from plant 2, no oxygen is added) (Table 3, Fig. 2):**

In control assays a fall in diversity could be observed at 24h after which it increased to reach the original values at 48h. Instead ciliate concentration decreased strongly along the period of experimentation.

At low concentrations of copper (1 and 2.5 ppm) diversity and ciliate concentration decreased for the first 24h in a similar way the control did (at 1 ppm there was even more concentration and diversity than in control). However at 48 h and 1ppm it was diversity what especially fell while ciliate concentration was slightly decreased but found at almost the same values of control. The effects of 2.5ppm of copper at 24h were already

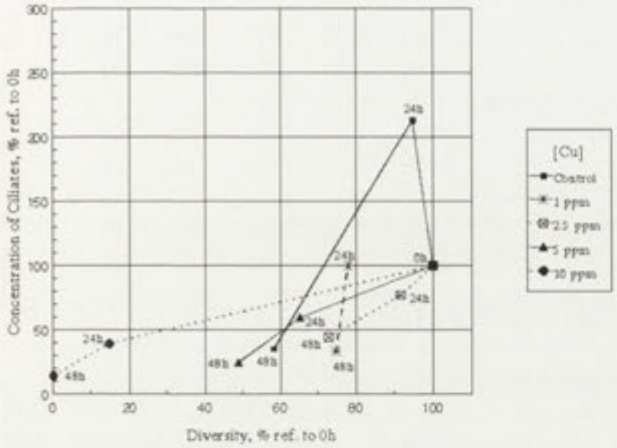


Fig. 4. Evolution of ciliate specific diversity of ciliates together with ciliate concentration in Experiment 3n (sludges taken from plant 3, no oxygen addition). Data are expressed as the percentage of the values for each corresponding parameter in the control bioassay at 0h

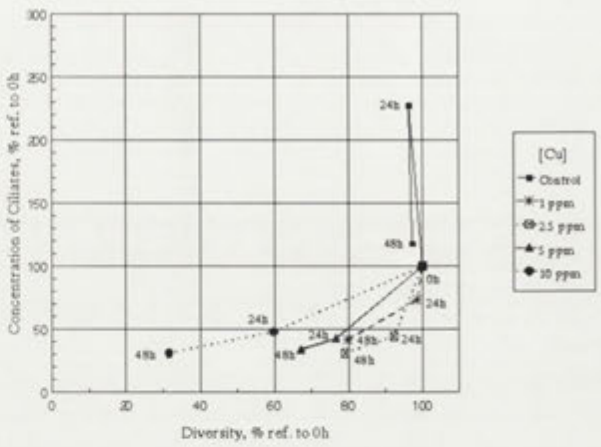


Fig. 5. Evolution of ciliate specific diversity of ciliates together with ciliate concentration in Experiment 3a (sludges taken from plant 3, oxygen was added). Data are expressed as the percentage of the values for each corresponding parameter in the control bioassay at 0h

clear both on diversity and ciliate concentration which had suffered an important decrease. At high concentrations of copper (5 and 10 ppm), both parameters fell to almost 0 yet at 24h.

**Experiment 3n (sludge from plant 3, no oxygen is added) (Table 4, Fig. 4):**

Control assays showed a strong growth of ciliates without any changes on the diversity until 24h after which a strong decrease on diversity and especially ciliate concentration was observed.

In assays intoxicated with 1 ppm it could be observed a decrease of diversity at 24h followed by a decrease in ciliate concentration at 48h. In assays with toxic concentrations above 1 ppm, diversity and ciliate concentration were gradually decreased along the experiment, decreases which were stronger as copper concentration was higher. It has to be pointed out however that at 48h diversity was higher in bioassay at 1 and 2.5 ppm of copper than control while the total number of ciliates remained quite the same.

**Experiment 2a (sludge from plant 2, oxygen is added) (Table 3, Fig. 3):**

In control assays diversity was slightly decreased with time while ciliate concentration was increased, especially at 24h.

The assays at 1, 2.5 and 5 ppm behaved like the control, finding an increase in total number of ciliates, the higher the concentration of toxic the lower this increase was. However diversity was a bit higher than control in assays at 1 and 2.5 ppm at 48h but ciliate concentration was lower. In the assays at 10 ppm both diversity and ciliate concentration were highly decreased.

**Experiment 3a (sludge from plant 3, oxygen is added) (Table 4, Fig. 5):**

In control assays the number of ciliates increased at 24h while at 48h it decreased, the specific diversity however remained the same along the experiment.

In the intoxicated assays either diversity and total number of ciliates decreased as copper concentration or toxic exposure time increased.

**Effects of copper on the different ciliate species and other groups of protozoa**

Bioassay showed that while the concentration of some ciliate species increased with the addition of copper, other died off completely. For instance, *Opercularia minima* from Experiment 2a (Table 3) at 5 ppm of Cu increased its concentration from 170 ind/ml (0h) to 280 ind/ml (24h) and still 475 ind/ml (48h).

On the other hand cyst formation was observed in *Opercularia coarctata* at 2.5 ppm (24h) which was a concentration slightly above its TLm (2.2 ppm Cu).

It was also observed that there was great variation in the toxic effects within the same species, depending on the plant from which the sludge was obtained (Tables 2, 3 and 4). Table 5 was drawn up to compare the toxic effects of Cu on ciliates from the three plants. In this table the species that were found in at least two of the three plants are represented. The TLm of these species at 24h and 48h were compared in the assays without addition of oxygen. The values of TLm, although approximate, since the concentration of individuals was subject to marked alterations, were significant enough for this comparison. It can be seen that the toxic effects

Table 4

Experiment 3n (Sludge taken from plant 3, no oxygen addition) and Experiment 3a (Sludge taken from plant 3, oxygen was added): Effects of different concentrations of Copper on the concentration of the different species of ciliates and other protozoa as well as on diversity in bioassay

| | | ppm of Cu | | | | |
|---|---|---|---|---|---|---|
| | Time | Contr. | 1 | 2.5 | 5 | 10 |
| Experiment 3n, organims in ind/ml | | | | | | |
| *Vorticella convallaria* | 0h | 205 | | | | |
| | 24h | 222 | 262 | 143 | 50 | 13 |
| | 48h | 13 | 33 | 37 | 0 | 0 |
| *Aspidisca cicada* | 0h | 106 | | | | |
| | 24h | 181 | 137 | 159 | 86 | 0 |
| | 48h | 63 | 83 | 137 | 60 | 0 |
| *Acineria uncinata* | 0h | 201 | | | | |
| | 24h | 389 | 194 | 174 | 250 | 243 |
| | 48h | 152 | 100 | 112 | 100 | 92 |
| *Epistylis plicatilis* | 0h | 137 | | | | |
| | 24h | 590 | 57 | 25 | 0 | 0 |
| | 48h | 0 | 0 | 0 | 0 | 0 |
| CILIATES TOTAL | 0h | 649 | | | | |
| | 24h | 1382 | 650 | 501 | 386 | 256 |
| | 48h | 228 | 216 | 286 | 160 | 92 |
| FLAGELLATES (x1000) | 0h | <1 | | | | |
| | 24h | <1 | <1 | <1 | <1 | <1 |
| | 48h | <1 | <1 | <1 | <1 | <1 |
| GYMNANOEBIA (x1000) | 0h | <1 | | | | |
| | 24h | <1 | <1 | <1 | <1 | <1 |
| | 48h | <1 | <1 | <1 | <1 | <1 |
| Experiment 3a, organims in ind/ml | | | | | | |
| *Vorticella convallaria* | 24h | 405 | 108 | 120 | 136 | 216 |
| | 48h | 308 | 114 | 90 | 140 | 172 |
| *Aspidisca cicada* | 24h | 202 | 67 | 23 | 0 | 0 |
| | 48h | 120 | 0 | 0 | 0 | 0 |
| *Acineria uncinata* | 24h | 270 | 175 | 56 | 86 | 66 |
| | 48h | 136 | 71 | 60 | 40 | 31 |
| *Epistylis plicatilis* | 24h | 600 | 127 | 90 | 56 | 30 |
| | 48h | 200 | 86 | 52 | 40 | 0 |
| CILIATES TOTAL | 24h | 1477 | 477 | 289 | 278 | 312 |
| | 48h | 764 | 271 | 202 | 220 | 203 |
| FLAGELLATES (x1000) | 24h | <1 | <1 | <1 | <1 | <1 |
| | 48h | <1 | <1 | <1 | <1 | <1 |
| GYMNANOEBIA (x1000) | 24h | <1 | <1 | <1 | <1 | <1 |
| | 48h | <1 | <1 | <1 | <1 | <1 |
| DIVERSITY OF CILIATES, bits/ind | | | | | | |
| Experiment 3n | 0h | 1.95 | | | | |
| | 24h | 1.85 | 1.52 | 1.79 | 1.27 | 0.29 |
| | 48h | 1.14 | 1.46 | 1.42 | 0.95 | 0.00 |
| Experiment 3a | 0h | 1.95 | | | | |
| | 24h | 1.88 | 1.92 | 1.80 | 1.49 | 1.16 |
| | 48h | 1.90 | 1.56 | 1.54 | 1.31 | 0.62 |

Table 5

Median tolerance limit (Tlm) to copper of the ciliate species common to at least two of the three plants

| | Experiment: | 1n | 2n | 3n |
|---|---|---|---|---|
| TLm, in ppm of Cu. (aprox. values) | | | | |
| *Vorticella microstoma* | 24h | 3 | 3.8 | - |
| | 48h | 0.7 | 2.3 | - |
| *Uronema marinum* | 24h | <0.5 | 2.5 | - |
| | 48h | <0.5 | 1 | - |
| *Aspidisca cicada* | 24h | <0.5 | - | 4.8 |
| | 48h | <0.5 | - | - |
| *Acineria uncinata* | 24h | 0.7 | - | >10 |
| | 48h | 0.45 | - | >10 |

decreased in these species in accordance with the plant the sludge had been taken from in the following order: Plant 1 > Plant 2 > Plant 3.

With regard to flagellates, their response was very different depending on the plant in which the sludge had been sampled. Thus, in Experiment 1a (Table 2) they grew in control assays while they almost disappeared after the addition of copper. However in Experiment 2n (Table 3) a fewer number of flagellates was found and its number remained almost unchanged in control assays while after intoxication its concentration slightly decreased. An increase in concentration of flagellates could be observed in Experiment 2a (Table 3) in bioassay with 0 (control) and 1 ppm of copper while in assays

with higher concentration of copper the number of flagellates decreased. In sludges from plant 3 (Experiments 3a and 3n) the number of flagellates was almost 0.

The concentration of gymnamoebia was very low in sludges used for Experiments 1n (Table 2), 3n and 3a (Table 4) and differences were hardly detected between control and intoxicated assays. A higher number of gymnamoebia was found in sludges from plant 2. In Experiment 2n (Table 3) the number of gymnamoebia was constant along the experience in control assays while it decreased as the concentration of copper and time of exposure to the toxic increased (except only for bioassay at 2.5 ppm of Cu at 24h). Despite the decreases found in Experiment 2n the addition of oxygen (Experiment 2a) allowed the growth of gymnamoebia in the presence of copper, growth that was higher as toxic concentration increased until a concentration of 10 ppm in which gymnamoebia decreased its number.

## DISCUSSION

As a general trend it can be observed that the toxic effects of copper are expressed as a decrease in specific diversity and total concentration of ciliates. Other parameters such as Tlm's of each species can also show toxic effects of copper.

However the study of toxicity in tests with ciliate communities is difficult with Tlm's because there is great variation in the sensitivity of the different species to the effects of copper, which influences the dynamics of protozoan communities. Thus those species that are more resistant to a given concentration of copper may enjoy reduced competition, and even increase their population, as can be seen for example in *Opercularia minima* from Experiment 2a (Table 3). Furthermore a similar effect can be observed in Experiment 1n in which an important increase in ciliate concentration is found which is mainly due to the growth of *Acineria uncinata* caused by the growth of flagellates this ciliate feeds on. These trophic phenomenons associated with toxicity produces sharp oscillations in the species' densities, and masks the calculation of specific TLm's.

The experiments showed that specific diversity of ciliates tends to decrease as the concentration of copper is increased (Figs. 1, 2, 3, 4 and 5) which had already been reported by Thomas and Seiber (1977), who worked with algae and other Protista. Specific diversity of ciliates can be used as a parameter of interest to evaluate the toxicity of Cu, since the variation in this parameter shows a pattern that is less subject to oscillation than the concentration of each species of ciliates (reflected in the Tlm's). However, as the definition of diversity does not take into account variations in the size of the population, we might find cases in which toxicity is expressed only as a limiting factor for the size of a population without detecting changes in diversity as it is the case of Experiment 3a at 1 ppm (between 24h and 48h, see Fig. 5). The reduction of the size of population may also in some cases result in changes in each species' frequency causing reduced decreases in diversity while the concentration of ciliates clearly falls, this case can be found in Experiment 2a (24h) (Table 3, Fig. 3) finding higher diversity at 10 ppm of copper than at 5 ppm of copper, however diversity finally fell in the bioassay at 10 ppm at 48h.

As suggested above concentration of ciliates can also be important when assessing the toxic effects of copper. But again since we are not taking into account diversity we are loosing important information about the complexity of the system as such is the case of Experiment 2n (Table 3, Fig. 2) at 1ppm (48h) in which the concentration of ciliates is the same than control assay while diversity is about 3 folds lower.

Thus, as a conclusion, both specific diversity and concentration of ciliates should be studied together as they become complementary when assessing toxicity on ciliate communities. Figs. 1 to 5 were drawn to show this, observing that depending on the plant the sludge had been taken from toxicity was expressed as a fall in at least one of the parameters (specific diversity or concentration of ciliates) but always tending to be expressed in both when toxic concentration is high.

However, in order to be able to use specific diversity and total concentration of ciliates to assess toxicity in ciliate communities, other factors that affect it should be taken into account. By this means, Ruthven and Cairns (1973) pointed out that toxic exposure time and dissolved oxygen did affect the toxic effects of heavy metals in protozoa.

Thus, specific diversity of ciliates decreased along our experiments which may be due to the toxic effects of copper but also the reduction in the organic load (BOD) which can be observed in control assays. As shown by Salvadó and Gracia (1991, 1993) at low organic loading there is a positive relation between BOD and ciliate diversity.

On the other hand, we have observed that the lack of oxygen addition becomes a limiting factor for the growth and survival of some protozoa, thus in control assays

corresponding to the experiments without oxygen addition, diversity and/or concentration of ciliates were found to be lower than in the experiment with oxygen addition. This effect can be seen comparing diversity of Experiments 2n with 2a and 3n with 3a (Figs. 2, 3, 4 and 5), being only a value at 48h (Experiment 2n) as exception. This effect was previously observed by Salvadó and Gracia (1992) who reported that the supply of dissolved oxygen increases specific diversity in different natural communities and in activated sludges. The importance of oxygen as limiting factor is also clearly shown by the mortality of some species, mainly peritrichs, under conditions of oxygen scarcity. Moreover, in experiments without oxygen addition the toxic effects of copper are expressed as an acceleration of mortality due to the lack of oxygen in some peritrich species as *Epistylis plicatilis* and *Vorticella convallaria* at 5 and 10 ppm (24h) (Table 4).

There is not only variation in the sensitivity of the different species to copper but also variations among the same species depending on the plant the sludge had been taken from, which is something clearly observed with Tlm's (Table 5) but also seen after detailed study of both specific diversity and ciliate concentration of intoxicated bioassay compared to control. This gradation found in toxicity suggests that there is an inverse relation between sludge age and toxicity of copper. Increasing the sludge age, according to Neujeed and Herman (1975) favors the retention of heavy metals in the biofloc, thus reducing its toxicity.

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# Melanin Revealed in Spores of the True Slime Moulds Using the Electron Spin Resonance Method

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**Summary.** The study was undertaken in order to characterize the type of pigment present in the spore wall of *Physarum polycephalum*, *Physarum nudum*, and *Fuligo septica*. The measurements were carried out on two kinds of material: crude spores and black pigment isolated from the spores using the electron spin resonance (ESR) method. The obtained results referring to the general character of the ESR spectra, width O.5 mT, and g-values appox. 2 definitely indicated the presence of the melanin-type pigment in all three species. Species variations found: 1) the intensity of the ESR signal from the crude spores was highest in *Ph. nudum*, and lowest in *F. septica*; 2) the ESR signal of the black pigment isolated from spores was highest in *Ph. polycephalum;* on the other hand, signal values of *Ph. nudum* and *F. septica* pigments were comparable with each other and were lower by about 1/3 than that of the signal value of spore pigment of *Ph. polycephalum*.

**Key Words**. Slime moulds, myxomycetes, spore pigment, melanin, electron spin resonance.

## INTRODUCTION

The true slime moulds (Myxomycetes) are simple eucaryotic organisms, in which fruit bodies with spores are produced as typical forms of the developmental phases in the complex life cycle of these organisms. Different groups of the slime moulds vary in the structure of their fruit bodies and spores and also in the spore colour. Pigmentation of the spores is one of the diagnostic features in the taxonomy of the Myxomycetes (Krzemieniewska 1960, Martin and Alexopoulos 1969, and others). Both biosynthesis of the pigment during plasmodia differentiation and the chemical nature of the spore pigment have hitherto been the subject of a few studies (Ward and Havir 1957, Daniel 1966, McCormick et. al. 1970, Chet and Hüttermann 1977, Chapman et al. 1983, Loganathan et al. 1988), some of which need to be reinvestigated. In general, melanin is assumed to be the spore wall pigment of the slime moulds. The characteristics were based on data obtained from pigment extraction, on various physical and chemical tests, and on the light absorption of the isolated pigment in ultraviolet and in the visible region of the spectrum. None of the tests and methods used till now are unequivocal as to the assignment of melanin to those pigments.

The aim of the present work was to introduce a new technique allowing the assessment of characteristics of

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the black pigment of spores of the slime moulds. The study deals with the spores of three species of the Physarales group, using the electron spin resonance method for pigment determination. It is hoped that the results obtained will open the way for further, detailed studies on the pigment in question, and the data may supply information on metabolic events in the plasmodium when developing fruit bodies, and on the role of light during these transformations.

## MATERIALS AND METHODS

Plasmodia of the true slime moulds *Physarum polycephalum* Schw., *Physarum nudum* Macbr., and *Fuligo septica* (L.) Wiggers were grown on mineral salt agar with rolled oat flakes sprinkled over it, in 10 cm diameter Petri dishes, at a temperature 22°C, in the dark, and were subcultured every 3 days. The method of culture was based on information given in the papers by Camp (1936), Hosoda (1980, 1981) concerning *Ph. polycephalum*, Rakoczy (1962) for *Ph. nudum*, and Rakoczy (1979) for *Fuligo septica*. Overgrown plasmodia, a few cm in diameter, were transferred onto Petri dishes with plain agar, and after 24 h incubation in the dark were next exposed to light until the fruit bodies had developed. Sporangia with spores of the slime moulds *Ph. polycephalum*, and *Ph. nudum*, and aethalia of *Fuligo septica* were collected, air dried, under sterile conditions, and gently sifted through a dense mesh strainer. Samples containing about 90% of spores (examination under the light microscope) were used as material for the investigations.

Black pigment was extracted from three 25 mg samples of spores of each species by boiling the material in 10 ml 1 M KOH distilled water for 2 h at 100°C (Chet and Hüttermann 1977) and centrifuged at 6000 x g for 10 min. The dark brown supernatant was collected and the pellet, still black, was rinsed with a fresh portion of KOH and centrifuged. The black residue was discarded and the combined supernatants were centrifuged at 10000 x g. The supernatant of each sample was brought to pH 3 with HCl. The resultant black precipitate was sedimented by centrifugation, washed with distilled water and centrifuged. Hydrolysing of the pigment was carried out with 5 ml of 7 M HCl distilled water for 2 h at 100°C. After washing with distilled water, the precipitate was dried in a desiccator to a constant weight.

The light source were miniature fluorescent tubes, cold white LF 6W, TELAM, Poland. The fluence rate of radiation on the level of the Petri dishes was 50 µmol $m^{-2}s^{-1}$. The radiation measurements were carried out using an I-Cor spectroradiometer, U.S.A.

The visible and ultraviolet absorption spectra of alkaline solutions of spore pigment were recorded using a Unicam SP 800 recording spectrophotometer.

All electron spin resonance (ESR) measurements were performed at X-band frequencies of about 9.5 GHz, at room temperature, using a Bruker ESR 300 spectrometer. The samples of the investigated materials were placed in Pasteur pipettes of the same bore diameter. The measurement conditions were: centre field 0.34 T, sweep field 5 mT, modulation amplitude 0.1 mT, time constant 82 ms, sweep time 84 s, microwave power 2 x $10^{-2}$ µW. The intensity of the ESR signals was calculated for 1 mg of the mass weight of the given sample. This made possible the comparison of the results for the three investigated species of slime moulds.

L-DOPA melanin was obtained from SIGMA, U.S.A., bacto-agar from Difco, Detroit, MI, U.S.A. All other chemicals were of analytical grade, Polish production, obtained from Polskie Odczynniki Chemiczne, Gliwice. Distilled water was obtained using a Corbabid - AQUA, distiller, Warszawa.

## RESULTS

Sporangia of the slime moulds *Physarum polycephalum, Physarum nudum*, and aethalia of *Fuligo septica* obtained in laboratory culture were well developed. The colour of the spores in mass was as follows: black in *Ph. polycephalum*, dull black in *F. septica*, and dark brown in *Physarum nudum*. In all species the spores were minutely spinulose. Microscopic inspection of the spores to be used in the investigations showed some contaminations with crushed capillitia, but no more than about 10% of the given spore sample. The situation did not change when the spores were washed with distilled water and with ethanol, therefore it was ignored.

The extraction of the pigment from the spores using organic solvents i. e. ethanol, acetone or chloroform did not give any positive results. The pigment was not elicited from the spores when using 1 or 3 M HCl of distilled water and keeping the samples at room temperature for 16 h or longer nor when boiling the spores with HCl for two or more hours. No differences in the species were found in this respect. Dark pigment produced by the species *Ph. polycephalum, Ph. nudum*, and *F. septica* and deposited in their outer spore wall was extracted with 1 M KOH distilled water more easily by boiling. Deep brown suspensions were obtained, centrifugation of which gave dark-brown supernatants and black pellets. Repeated extraction of the pellet in the same conditions released a small amount of pigment (the supernatant was brownish), therefore the pellet was washed only with a small portion of 1 M KOH. Precipitation of the pigment occurred when the supernatant was acidified to pH 3. The time required for pigment precipitation showed no distinct differences between species and sedimentation of the pigment was usually completed within 3 to 4 min. The macroscopic appearance of the spore pigment of the investigated species was black and in *Physarum nudum* tiny granules of pigment prevailing as compared with *Ph. polycephalum* and *F. septica* in the samples of which well distinguishable granules were present. Pigment isolated

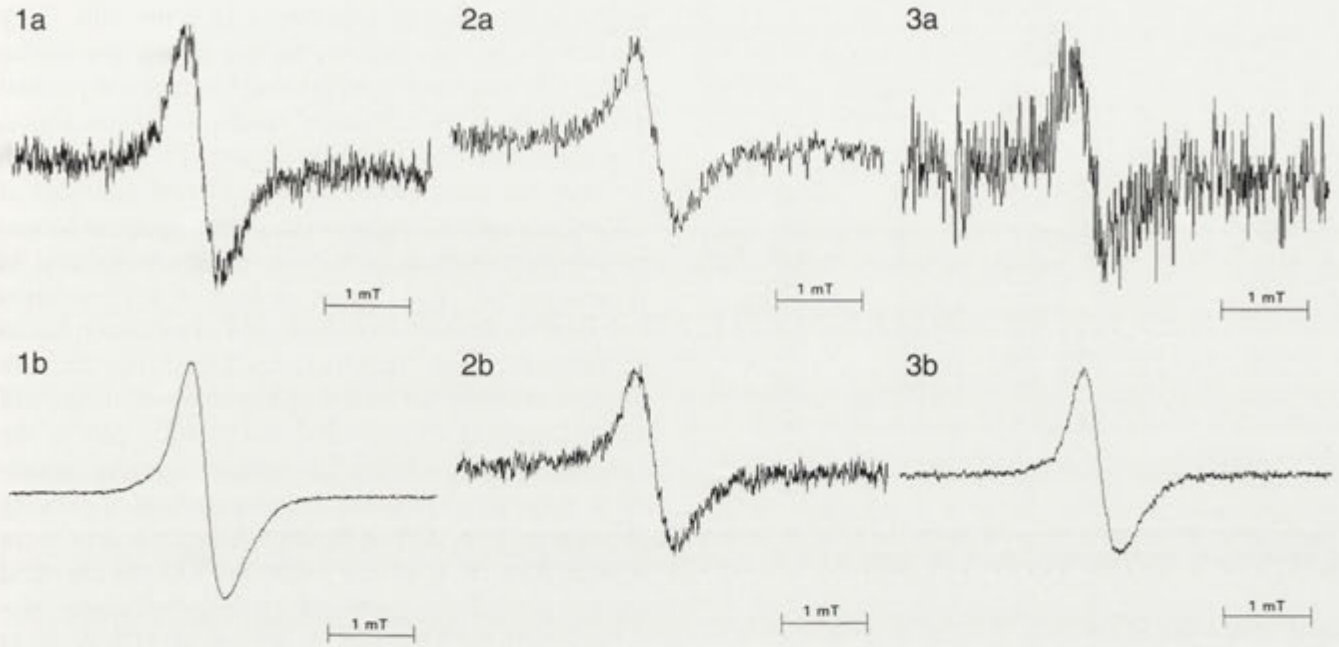


Fig. 1. ESR signal of spores (A) and of black pigment from the spores (B) of *Physarum polycephalum*. Measurement conditions: frequency 9.5 GHz, centre field 0.34 T, sweep of field 5 mT, modulation amplitude 0.1 mT, time constant 82 ms, sweep time 84 s, microwave power $2x10^{-2}$ μW, room temperature. Curve "A" with relative receiver gain 20. Curve "B" with relative receiver gain 4

Fig. 2. ESR signal of spores (A) and of black pigment isolated from the spores (B) of *Ph. nudum*. Measurement conditions as in Fig. 1. Curve "A" with relative receiver gain 10. Curve "B" with relative receiver gain 5

Fig. 3. ESR signal of spores (A) and of black pigment from the spores (B) of *Fuligo septica*. Measurement conditions as in Fig. 1. Curve "A" with relative receiver gain 400. Curve "B" with relative receiver gain 5

from the spores of all three species was soluble in alkali, insoluble in water, HCl, and organic solvents (acetone, chloroform, ethanol, methanol), and gave positive reactions (decolorization) to an oxidizing agents, e. g. $H_2O_2$, NaOCl. The solubility of the pigment in alkali, and the time required for complete precipitation after acidification of the solution, revealed no species differences which should be taken into consideration. The absorption of light of alkaline solutions of the pigment exhibited no characteristic peaks in UV and VIS regions of the spectrum.

The electron spin resonance (ESR) measurements carried out for both the spores and the black pigment isolated from the spores of *Ph. polycephalum, Ph. nudum*, and *F. septica*, indicated the presence of a substance giving an ESR spectrum with the following parameters: width 0.5 mT, g-value approx. 2. The shape of the ESR spectrum curves, and the values of their parameters (Figs. 1 to 3) indicated the melanin-type of black pigment present in the slime moulds *Ph. polycephalum*, *Ph. nudum*, and *F. septica*. The features of the ESR spectra were almost identical in each of the three species, and also with those obtained for DOPA-melanin measured in conditions analogous to those of the investigated materials. This finding also pointed to the melanin character of the black pigment deposited in the outer spore wall of the myxomycetes. The data in Figs. 1 to 3 present the relative intensity of the signals for each species. To enable the comparison of the data of all the studied species, their ESR signals were normalized per 1 mg of the sample. The calculations are presented in Table 1 and in Fig. 4. Analysis of the data revealed the existence of two types of difference, both of quantitative nature, in relation to the species: (1) the intensity of the ESR signal of crude spores was highest in *Ph. nudum* and lowest in *F. septica* (Fig. 4 and Table 1); (2) the intensity signal of the black pigment from the spores was highest in *Ph. polycephalum*, while the signal values of *Ph. nudum* and *F. septica* pigments were comparable to each other and equal to about two thirds of the intensity value obtained for the pigment isolated from the spores of *Ph. polycephalum* (Fig. 4, Table 1). The amounts of black pigment, calculated as the percentage of the air-dried weights of the spores of *Ph. polycephalum, Ph. nudum*, and *F. septica* were 16.1, 12.3, and 10.8 respectively. It seems however, that the true values are probably higher, because the remainders after pigment

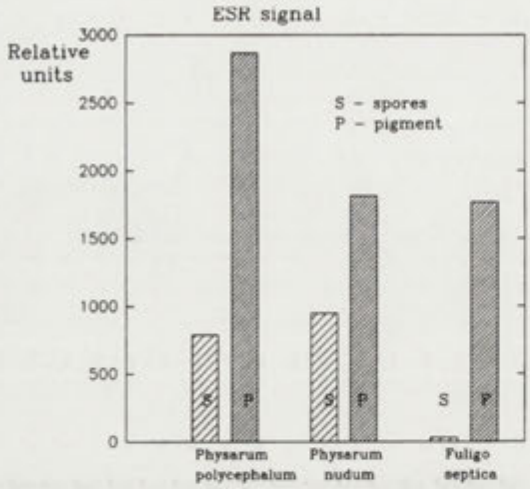


Fig.4. ESR signal intensity of spores and of black pigment isolated from the spores (calculated per 1 mg of the sample weight)

Table 1

Intensity of the ESR signals (calculated per 1 mg of sample weight) from the spores and from the black pigment isolated from the spores of the true slime moulds

| Species | ESR signal intensity | |
|---|---|---|
| | Spores | Pigment |
| *Physarum polucephalum* | 790 | 2866 |
| *Physarum nudum* | 948 | 1813 |
| *Fuligo septica* | 30 | 1766 |

extraction were still black. This might indicate that a certain amount of melanin was still present in the spores, which was not extracted during the procedure used. Evidence of this was found during the initial attempts on detailed analysis of the melanin pigment from the spores of *Ph. nudum* using a different method of pigment extraction (under investigation). The ESR signal obtained was quite pronounced and much higher than the one presented here. It has be supposed that there exist species variations in melanin localization with respect to different substances present in the outer spore wall which enclosed and more or less masked the melanin pigment.

## DISCUSSION

Melanins are a class of natural pigments produced by a wide variety of the organisms. Although considerable research has been carried out on melanins isolated from animal tissues, from fungi and other organisms, the literature pertaining to dark pigments in the Myxomycetes is scarce. Ward and Havir (1957) were the first to suggest that the black pigment produced in *Physarum polycephalum* during sporulation is a melanin. They studied the enzyme activity in sporulating plasmodia, which they assumed to be involved in melanin production. However, the presented results are inconclusive. The studies by Chet and Hüttermann (1977) dealt with the enzymes and phenols in two different materials of *Ph. polycephalum*: in plasmodia during spherule formation (which is induced by starvation alone), and in sporulating plasmodia, i.e. in the stage of differentiation into fruit bodies (for sporulation the illumination has to be followed by starvation). They found that the dark pigment is connected only with the phase of maturity of the sporangia in this species, and is not a part of the morphogenetic processes, i. e. appearing during sporangia or spherules formation. In spherules and in growing plasmodia of *Ph. polycephalum* the dark pigment is not present. Two reports were concerned with the chemical composition of the spores of *Ph. polycephalum* (McCormick et al. 1970) and *Fuligo septica* (Chapman et al. 1983). An interesting study on the nature of spore wall pigments in a few species of the orders Stemonitales and Physarales and one species from Liceales was published by Loganathan et al. (1988). In all the works the spore wall pigment was identified on the basis of the extraction method, absorption spectra in UV and VIS regions of light, and on the results of the diagnostic tests according to Thomas (1955). None of the identification methods is specific for melanin compounds. Though all the investigators used the name "melanin" for the spore wall pigment of the myxomycetes, it might be regarded as presumption.

The aim of the present work was to obtain sufficient evidence for the statement that the black pigment in the spores of the slime moulds *Physarum polycephalum*, *Physarum nudum*, and *Fuligo septica* is a melanin-type compound. For this reason it was necessary for the study to be carried out on material comparable with that used by previous investigators. Accordingly, identical methods for pigment extraction and purification were used and the same diagnostic tests were carried out.

Both the pigment present in crude spores and isolated from the spores (Figs. 1 to 3) give ESR signals with strikingly similar parameters, with respect to the curve shape, width, and g-value, to those found for the synthetic DOPA-melanin as well as natural melanin derived from different materials (Commoner et al. 1954, Mason et al. 1960, Blois 1966, Blois et al. 1964, Sealy et al. 1982, Prota 1988). A weak ESR signal of similar parameters can be observed in the cells or tissues of

intense metabolism, but the signal disappears when the cells are killed, e.g. with high temperature. This was not found in the spores of the studied species of the myxomycetes.

Spin resonance spectroscopy represents one of the special physical methods guaranteeing that the results obtained by its application under defined conditions permit the unequivocal statement that the given substance is or is not a melanin-type compound. The ESR method can be used not only to study melanin produced *in vitro* or isolated from biological materials, but also that of pigmented cells from the cell cultures of plant, animal, or tumour tissues, and even to quantify melanin in pigmented biological materials (Sarna and Łukiewicz 1972, Pilas and Sarna 1985).

Although it is not yet possible to classify the melanin pigment present in the spore wall of the slime moulds as one belonging to a recognized group of these compounds, the obtained findings, it is hoped, should contribute to further detailed studies of the chemical nature of melanin in these organisms.

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# Ultrastructural Localization of Enzymes in *Entamoeba histolytica*. I. Acid Phosphatase, Alkaline Phosphatase and Thiamine Pyrophosphatase

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**Summary.** Histochemical studies on *Entamoeba histolytica* indicate that there is not only morphological but also enzymatic differentiation of intranuclear bodies. These were found to contain acid phosphatase, alkaline phosphatase and thiamine pyrophosphatase activity. Cytoplasmic grains that stained strongly for the three enzyme activities were associated with helicoidal structures. Amoebae obtained from a host contained more grains and helices than amoebae cultured in vitro. As the membranes and vesicles displaying thiamine pyrophosphatase activity were localized in the vicinity of nucleus, they may be an analogue of the Golgi apparatus in Metazoa. Additional observations provide evidence that the vacuoles aggregated under the plasmalemma have the digestive function and that a surface structure that has been denoted as "trigger organ" takes part in extracellular secretion of the contents food-vacuole derived vesicles.

**Key words.** *Entamoeba histolytica*, phosphatases.

## INTRODUCTION

Previous studies have shown that the ultrastructure of amoebae of *Entamoeba* genus accommodate specific organellae, whose function has not hitherto been fully understood. Ludvik and Schipstone (1970), Rosenbaum and Wittner (1970), Feria-Velasco and Trevino (1972), Proctor and Gregory (1972), Zaman (1973) as well as Chernov et al. (1984) described intranuclear bodies, however the interpretations given by the authors concerning the origin and functions of these structures are not univocal.

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Ludvik and Schipstone (1970), Rosenbaum and Wittner (1970) as well as Proctor and Gregory (1973) recorded in *E. histolytica* cytoplasm the presence of helicoidal structures, which, as the authors suppose, are of polyribonucleic character.

Controversial is also the function of the group of vacuoles localized under cup-like invaginated plasmalemma with digital process - the "trigger organ", described by Eaton et al. (1969, 1970) in *E. histolytica* obtained from colon of a patient with symptomatic dysentery. In the membranes of these vacuoles the authors detected the activity of acid phosphatase, which provided the basis to consider them as specific lysosomal system of Protozoa, having been defined by them as lysosomal surface, and the digital process as "trigger organ". Since Eaton et al. (1969, 1970) observed both the lysosomal surface and the "trigger organ" ex-

clusively in trophozoites *E. histolytica* obtained from intestine,they ascribed to these structures the direct share in attacking the host's tissues. The theory was put in doubt by studies carried out by Proctor and Gregory (1972, 1973), who discovered the lysosomal surface not only in amoebae taken directly from the host, but also in amoebae from in vitro culture, moreover the studies conducted by Rondanelli et al. (1974a, b) disclosed the lysosomal surface in trophozoites of *Entamoeba coli* and *Entamoeba moshkovskii.*

The aim of the present paper is to provide additional pieces of information that would be able to elucidate the origin and function of these specific *Entamoeba* organellae.

## MATERIALS AND METHODS

The studied strain PS-2 of *Entamoeba histolytica* isolated from feces of a patient with symptomatic amebiasis was cultured on MSF medium with mixed bacterial flora (Raeves et al. 1957).

Guinea-pigs weighing 240-250 g were inoculated intracecally with suspension 2-2.5 x $10^5$ amoebae from logarithmic phase of culture growth. Amoebae used for infecting the animals and for cytochemical examinations were obtained by centrifuging the culture at the speed of 2000 x g.

Fourteen days post the infection, the guinea-pigs were dissected under ether narcosis and the content of the cecum was collected from the infected animals. Amoebae from intestine were obtained by manifold decantation of intestinal content in Ringer's solution.

Amoebae designed for cytoenzymatic examinations were fixed 10 min with glutaraldehyde, and subsequently washed with cacodylic buffer (Karnovsky 1965).

Reaction to acid phosphatase was carried out in incubation solution containing 50 mM acetate buffer pH 5.0, 10 mM sodium β-glycerophosphate and 4 mM lead nitrate (Gomori 1953). After 30 min the reaction was stopped by rinsing with water followed by cacodylic buffer. A control reaction was carried out with 100 mM NaF as acid phosphatase inhibitor.

The reaction to alkaline phosphatase was allowed to take place in incubation solution containing 200 mM propanediol buffer pH 9.4, 15 mM sodium β-glycerophosphate, 200 mM calcium chloride and 160 mM magnesium sulfate (Wetzel et al. 1967). After a 30 min incubation, amoebae were rinsed with water and placed for 3 min in 70 mM cobalt nitrate. The controls were incubated without substrate.

In order to detect thiamine pyrophosphatase, amoebae were incubated in a solution containing 200 mM tris-maleate buffer pH 7.0, 100 mM thiamine pyrophosphate chloride, 250 mM manganic chloride and 30 mM lead nitrate (Novikoff and Goldfinger 1961). After 60 min the reaction was stopped by rinsing amoebae with water and followed to cacodylic buffer. A control reaction was performed in incubation mixture without substrate.

After the use of each solution, the amoebae were centrifuged for 5 minutes at 2 000 x g. After dehydration through graded alcohols the samples were embedded in Spurr resin. Ultrathin sections were treated for 10 min with 2% uranyl acetate, and observed in electron microscope Jeol-Jem-1200 EX.

## RESULTS

Nuclei of *E. histolytica* contain peripheral agglomerates of bodies with diameters between 0,01 μm and 0,2 μm, which are surrounded by a single or double membrane. A subset of these bodies, with a diameter of about 0,05 μm, contained acid phosphatase activity (Fig. 1). The product of reaction to this enzyme fills them completely or exists exclusively in the membranes surrounding them.

In single intranuclear bodies with diameter of approx. 0,01 μm reaction of thiamine pyrophosphatase was found (Fig. 2). Very strong reaction of alkaline phosphatase was found in the biggest of the intranuclear inclusions, with diameter of approx. 0,2 μm (Fig. 3).

Comparing amoebae cultured in vitro with amoebae obtained from intestine of infected guinea-pigs, no differences were revealed either in the count of intranuclear bodies or in the enzymatic reactions products accumulated in them.

In cytoplasm of trophozoites of *E. histolytica* from in vitro cultures, and from intestine of the infected animals, it was observed that there were accumulations of helicoidal structure, frequently in close vicinity of food vacuoles (Fig. 4). These helices are distributed unevenly over the area of cells. Some regions of cytoplasm are characterized by exceptionally large amount of the structures. Helices assume the form of the rugose threads or they are accompanied by regularly packed, of uniform size, grains with diameter of 0,05 μm, which display a positive response to the studied enzymatic reactions (Fig. 4).

Figure 4 depicts the grains shifting from helices to the area of cytoplasm and simultaneously their partial decondensation. Despite the presence of helices and helices with grains disclosed both in amoebae from in vitro culture, and intestine of guinea-pigs, however, their larger number was observed in trophozoites obtained from infected animals.

In membranes of food vacuoles (Fig. 5) as well as membranes of vacuoles that are localized under the cup-like recessed plasmalemma (Fig. 6) a strong reaction of acid phosphatase was found.

Vacuoles surrounded by double membrane and wrapped by digital process (trigger organ) indicated a weak reaction to alkaline phosphatase (Fig. 7). Vacuoles of similar size, being also surrounded by double membrane and containing the product of reaction of alkaline phosphatase, were also observed outside the cell (Fig. 7).

In small vacuoles with diameter of about 0.05 μm, neither enzymatic reactions nor electron dense content was encountered.

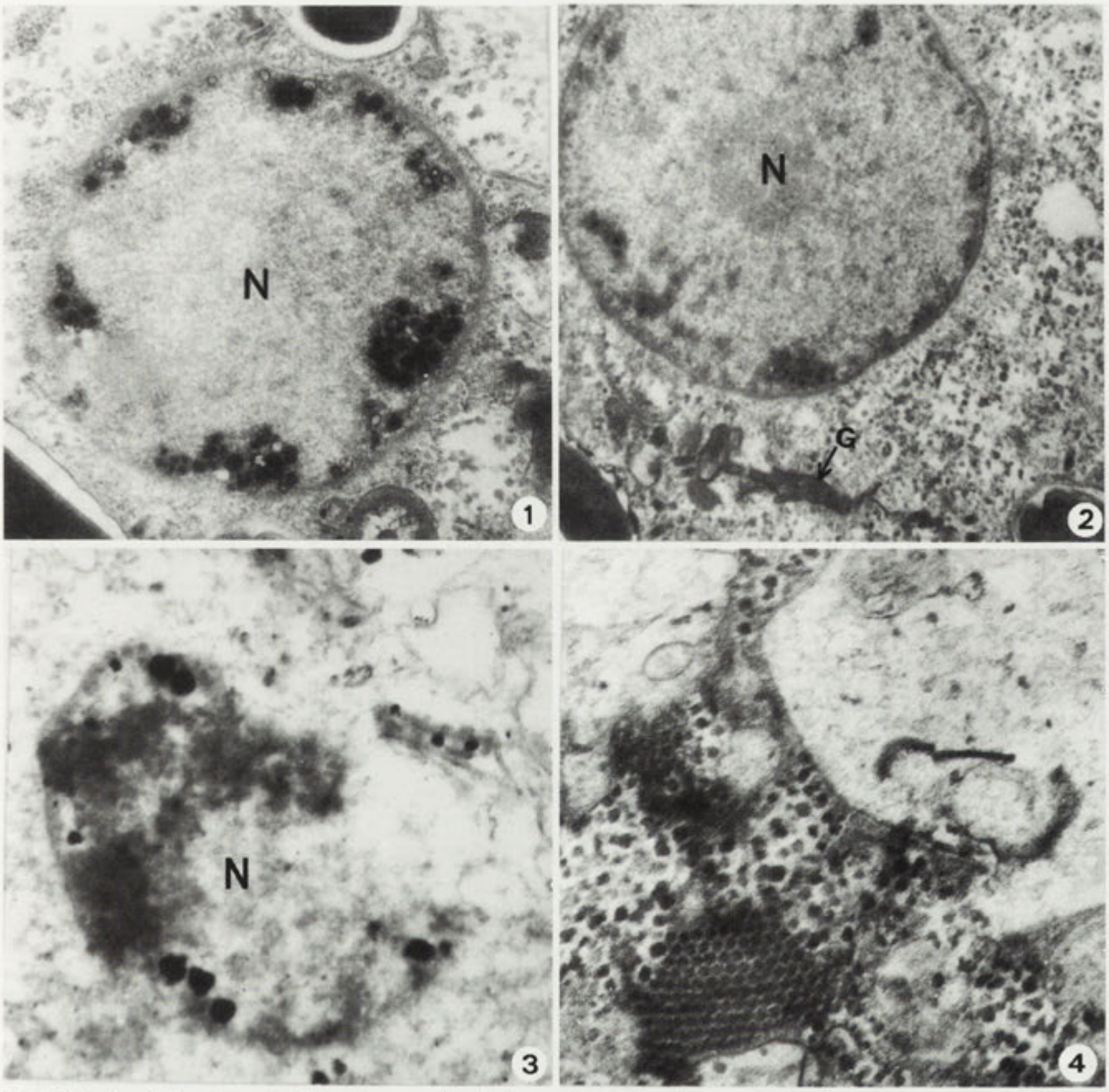


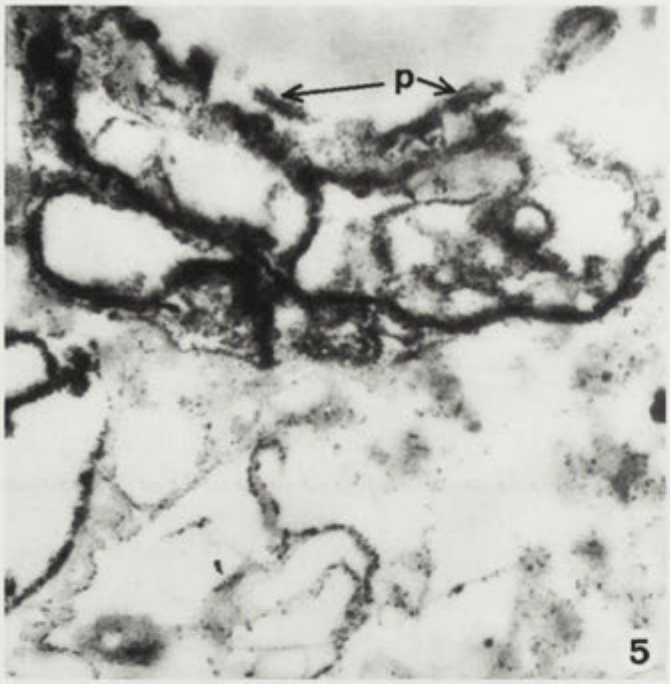

Fig. 1. Histochemical reaction of acid phosphatase activity in intranuclear bodies of *Entamoeba histolytica* from the colon of an infected quinea-pig. x 20 000 N - nucleus
Fig. 2. Histochemical reaction of thiamine pyrophosphatase activity in intranuclear bodies and Golgi-like apparatus of *Entamoeba histolytica* from the colon of an infected quinea pig. x 20 000 N - nucleus, G - Golgi-like apparatus
Fig. 3. Histochemical reaction of alkaline phosphatase activity in intranuclear bodies of *Entamoeba histolytica* from the colon of an infected quinea pig. x 20 000 N - nucleus
Fig. 4. Histochemical reaction of alkaline phosphatase activity in granules dispersed in cytoplasm and agglomerated in polyribosomal region helices of *Entamoeba histolytica* from the colon of an infected quinea pig. x 25 000

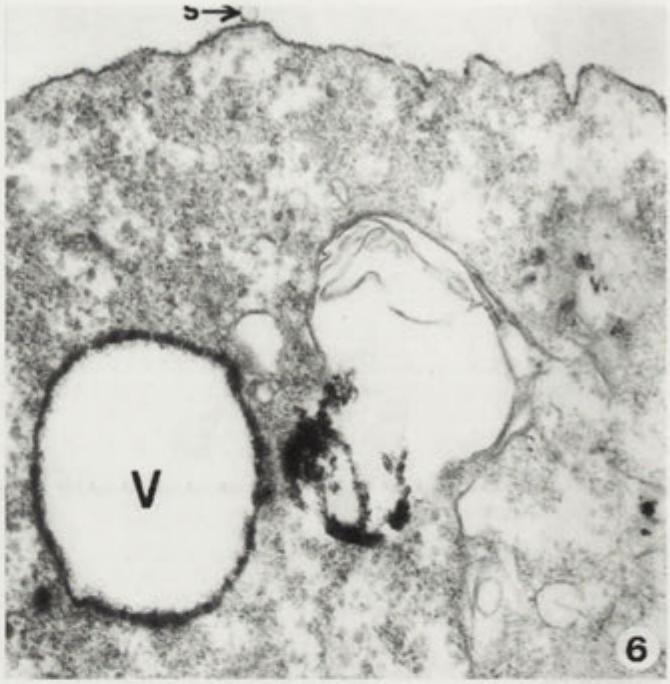
Some membranes and vesicles appearing in the vicinity of nucleus show the thiamine pyrophosphatase activity (Fig. 2).

The arrangement of these membranes and vesicles resembles the Golgi apparatus.

## DISCUSSION

The results of the performed histochemical studies in *Entamoeba histolytica* disclosed not only morphological also functional differentiations of intranuclear bodies.



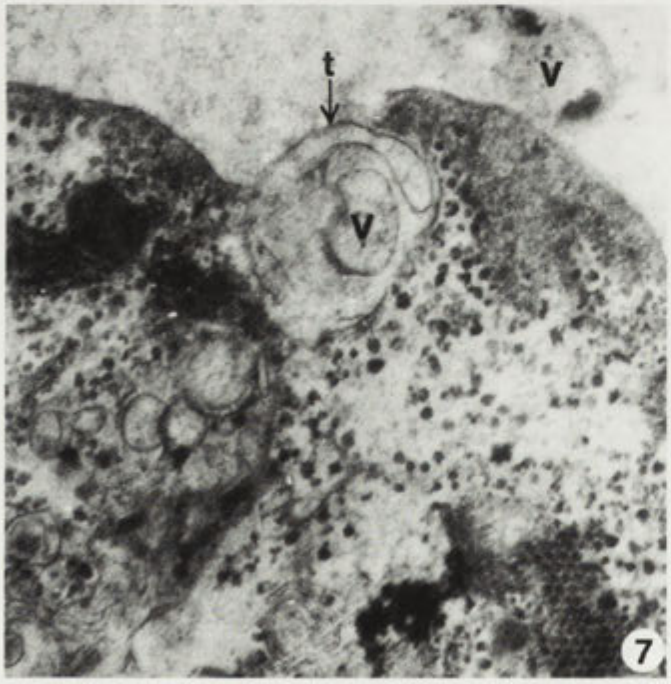




Fig. 5. Strong reaction of acid phosphatase activity in vacuoles localized under the plasmalemma. x 15 000 pl - plasmalemma
Fig. 6. Reaction of acid phosphatase in membrane of food vacuole. x 15 000 s - small vacuole without deposit of enzymatic reaction
Fig. 7. Grains with deposit of alkaline phosphatase reaction's dispersed in cytoplasm and agglomerated on helices. Weak reaction of this enzyme in the vacuole wrapped by "trigger organ" and in the vacuole localized outside cell. x 20 000 V - vacuole, t - "trigger organ"

Their origin is hard to elucidate. Ludvik and Shipstone (1970) suggest that they may be virus-like structures. However, the enzymatic activity present in them exclude entirely such a possibility. It may be assumed that they are derived from cytoplasm and constitute part of the lysosomal system of *E. histolytica*.

The provided studies suggest that the cytoplasmic grains with diameter of approx. 0.05 µm displaying activity of acid phosphatase, alkaline phosphatase and thiamine phosphatase originate from complexes of helicoidal structures. Possibly, enzymatic proteins are synthesized on helices, from where they proceed to the cytoplasm. The localization of helicoidal complexes in the immediate vicinity of nutritive vacuoles suggests that substrates for the synthesis of proteins are delivered from nutrients digested in the vacuoles. The role of helices in the biosynthesis of proteins would be in agreement with the suggestions of Ludvik and Schipstone (1970), Proctor and Gregory (1972, 1973) as well as Rosenbaum and Wittner (1970) pointing out the

polyribosomal character of the said structures. More numerous agglomerations of helices and grains accompanying them in amoebae originating from intestine of infected animals may reflect more intensive metabolism therein as compared to amoebae cultured in vitro. The intensification of metabolism is sure to be the consequence of increased phagocytosis and vacuolization in *E. histolytica* living in the intestine.

Studies hitherto performed failed to reveal the presence of Golgi apparatus in amoebas of Entamoeba genus. The actual studies exposed in *E. histolytica* the thiamine pyrophosphatase activity in membranes and vesicles, whose pattern resembles Golgi apparatus. Since this enzyme is a marker of Golgi apparatus, its presence is evidence of Golgi-like character of the observed structures in this Protozoa. Vesicles of almost similar size filled with product of reaction to thiamine pyrophosphatase appearing in the area of cytoplasm and in the neighborhood of plasmalemma are likely to originate from Golgi-like apparatus.

It seems that metabolic products in *E. histolytica* are being secreted or excreted in the form of small and large vacuoles differing as to their content. Small vacuoles, diameter 0.05 μm, without electron dense content and without enzymatic reactions detach themselves most probably from plasmalemma, while vacuoles outside amoebae with diameter of 0.04 μm and exhibiting a weak response to the reaction to alkaline phosphatase, originate from vacuoles accumulating under recessed plasmalemma, from where they are "thrown out" outside the cell by means of "trigger organ". This finding is proven by similarly sized vacuoles, whose reaction to alkaline phosphatase is weak too, being wrapped by "trigger organ". Other food vacuoles often showed numerous smaller vacuoles, which, it should be assumed, contain digested products of amoebae designed later for extracellular secretion.

In *E. histolytica* obtained from intestine of infected animals secretive vacuoles outside the cell were more numerous than in amoebas cultured in vitro.

The term lysosomal surface used by other authors Eaton et al. (1969, 1970), Proctor and Gregory (1972, 1973), Rondanelli et al. (1974a, b), for defining the cohort of vacuoles localized under plasmalemma is not justified. Since in their membranes, like in the membranes of nutritive vacuoles, there is strong reaction to acid phosphatase, they should be considered to be also food vacuoles. The accomplished studies made it possible to pinpoint the individual stages in the extracellular secretion of vacuoles, those stemming food vacuoles, inception of secretive vacuoles in the area of food vacuoles, their removal outside the cell by the aid of trigger organ, and their presence already outside the cell. The membranes of secretive vacuoles are formed from the food vacuoles, which is substantiated by their double uniform structure. The fact that vacuoles observed outside the cell are originated in the area of food vacuoles is additionally supported by the finding that the same response of the reaction to acid phosphatase is present in secreting vacuoles existing still in food vacuoles, and in those being outside the cell. However, the performed studies do not allow elucidation of the character of the content in these vacuoles. Since the secretive vacuoles were encountered both in amoebae stemming from intestine of infected animals and from amoebae cultured in vitro, there is no basis to ascertain that they have some relationship with pathogenesis of amebiasis.

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# Study of Some Marine Fish Coccidia of the Genus *Eimeria* Schneider, 1815 (Apicomplexa, Coccidia) from Senegal Coasts

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**Summary**. Eleven coccidia of the genus *Eimeria* are described from marine fishes in Senegal. These are. *Eimeria adioryxi* sp. n. from *Adioryx hastatus*; *E. ethmalosae* sp. n. from *Ethmalosa fimbriata*; *E. gabonensis* sp. n. from *Chelidonichthys gabonensis; E. kayarensis* sp. n. from *Raja miraletus*; *E. perciformis* sp. n. from *Pomadasys incisus* and *Epinephelus goreensis*; *E. ryptici* sp. n. from *Rypticus subbifrenatus*; *E. syacii* sp. n. from *Syacium micrurum; E. ashburneri* Molnar and Rohde, 1988 from *Pagellus bellottii* and *Sparus caeruleostictus*; *E. catalana* Lom and Dykovà 1981 from *Bodianus speciosus* and *Scarus hoefleri*; *E. dakarensis* Faye, 1988 from *Cephalopholis taeniops* and *Epinephelus alexandrinus*; *E. ivanae* Lom and Dykovà, 1981 from *Serranus cabrilla* and *Serranus scriba* and *E. sardinae* (Thélohan, 1890) Reichenow, 1921 from *Sardinella aurita* and *Sardinella maderensis*. These coccidia infect the digestive tract and the testis of their hosts and have been found in fishes belonging to eleven families.

**Key words**. Marine Fish Coccidia - *Eimeria ashburneri, E. catalana, E. dakarensis, E. ivanae*; new species - *Eimeria adioryxi, E . ethmalosae, E. gabonensis , E. kayarensis, E. perciformis, E. ryptici, E. syacii.*

## INTRODUCTION

Hitherto, to our knowledge, twenty coccidia from African fishes have been reported (Fantham 1932; Landsberg and Paperna 1985, 1987; Obiekezie 1986; Faye 1988; Diouf 1993; Diouf and Toguebaye 1993). Among these coccidia only seven species belong to the genus *Eimeria.* These are: *Eimeria clini* Fantham, 1932; *E. gobii* Fantham, 1932; *E. vanasi* Landsberg and Paperna, 1987; *E. dakarensis* Faye, 1988; *E. parapristipomae* Faye, 1988; *E. sparidae* Faye, 1988 and *Eimeria* sp. Faye, 1988 parasiting the anterior part of the intestine of *Boops boops.* Recent investigations of marine fishes from senegalese coasts have revealed infections with coccidia belonging to the genus *Eimeria.* This paper is a taxonomic study of these coccidia.

## MATERIALS AND METHODS

Over 8000 marine fishes collected, from the coasts of Senegal were examined. They belong to 23 orders, 78 families, 131 genera and 181 species. Fresh smears from all organs were conducted for light microscopy to look for coccidia. Oocysts and sporocysts were measured and drawn from freshly caught samples. For scanning electron microscopy (SEM), fragments of infected tissues were smeared on round coverglasses, air dried and fixed for 24 h, at 4°C, with 2.5% v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. The smears were then post-fixed for 1 h with 1% (w/v) osmium tetroxide

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in cacodylate buffer at 4° C. After washing in the same buffer and dehydration in progressive ethanol series and pure acetone, the material were subjected to $C0_2$ critical point drying, coated with gold and observed in a Jeol 35 CF.

## RESULTS

### *Eimeria adioryxi* sp. n. (Figs. 1, 2, 30)

Host: *Adioryx hastatus* Valenciennes, 1829 (Bericiformes: Holocentridae).

Site of infection: intestine.

Prevalence: 20% (1/5).

Description: oocysts are spherical and measure 9.7 ± 0.6 µm in diameter. Oocyst residuum is absent but one polar granule, measuring 1.3 ± 0.3 µm in diameter, is present ( Figs. 1, 30 ). Each mature oocyst contains four ovoid sporocysts, 6.8 ± 0.8 µm long by 5.1 ± 0.3 µm wide. Stieda body consisting of a flat thickening at one end of the sporocyst (Figs. 2, 30). Sporocyst residuum occurring in the form of four refringent granules. Two sporozoites arranged in the shape of a cross are present in each mature sporocyst (Fig. 30).

Remarks: compared with the other *Eimeria* species which oocyst diameter is lower than 10 µm and possessing one polar granule, the species described here resembles *Eimeria pleurostici* Molnar and Rohde, 1988. Nevertheless it differs from this species by the composition of the sporocyst residuum, the arrangement of sporozoites within the sporocysts and by the fact that *E. pleurostici* has an exogenous sporulation (Molnar and Rohde 1988a). We consider this a new species and we propose the name *Eimeria adioryxi* from the generic name of its host.

### *Eimeria ashburneri* Molnar and Rohde, 1988 (Figs. 3, 4, 5, 31)

Hosts: *Pagellus bellottii* Steindachner, 1882 (Perciformes: Sparidae) and *Sparus caeruleostictus* (Valenciennes, 1830) (Perciformes: Sparidae).

Site of infection: intestine.

Prevalence: 20% (12/60) for *P. bellottii* and 7.8% (5/64) for *S. caeruleostictus*.

Description: oocysts containing sporoblasts were observed (Fig. 3). Mature oocysts spherical, 8.5 ± 0.6µm in diameter. One polar granule measuring 0.4 ± 0.1 µm in diameter has been observed (Figs. 4, 31) but oocyst residuum is absent. Sporocysts ovoid, 5.9 ± 0.6 µm x 3.9 ± 0.4 µm, Stieda body knoblike, at the tapered end of the sporocyst (Figs. 5, 31). Several small granules form the sporocyst residuum (Figs. 5, 31). Each of the mature sporocysts contains two falciform sporozoites arranged in the shape of a cross (Fig. 31).

Remarks: *Eimeria* species have been described from fishes belonging to Sparidae family. *Eimeria sparidae* from *Sparus aurata*, *E. maggiae* from *Pagellus erythrinus* and *E.smaris* from *Spicara smaris* (Lom and Dykovà 1981, Faye 1988, Daoudi et al. 1989) but none of them resembles the species described here. When we compare it with the other *Eimeria* species known from fish, it is similar to *Eimeria ashburneri*, parasiting *Macquaria ambigua*, a freshwater fish (Molnar and Rohde 1988b).

### *Eimeria catalana* Lom and Dykovà, 1981 (Figs. 6, 7, 8, 32)

Hosts: *Bodianus speciosus* (Bowdich, 1825) (Perciformes: Labridae) and *Scarus hoefleri* (Steindachner, 1882) (Perciformes: Scaridae).

Site of infection: intestine.

Prevalence: 33.3% (2/6) for *B. speciosus* and 20% (1/5) for *S. hoefleri*.

Description: spherical zygotes having the same diameter than mature oocysts were observed (Fig. 6). Oocysts spherical measuring 10.8 ± 0.5 µm in diameter. Oocyst residuum absent. Three to six polar granules are present (Figs. 7, 8). Sporocysts ovoid, 6.1 ± 0.3 µm x 4.7 ± 0.3 µm. Stieda body present (Figs. 8, 32). Two falciform sporozoites are present in each mature sporocyst (Figs. 7, 32). Sporocyst residuum consisting of about 10 refringent granules (Figs. 7, 32).

Remarks: to our knowledge six *Eimeria* species have been described from fishes belonging to Labridae family. These are: *Eimeria catalana* Lom and Dykovà, 1981; *E. banyulensis* Lom and Dykovà, 1982; *E. roussillona* Lom and Dykovà, 1981; *E. petrovitchi* Daoudi, 1987; *E. variabilis* Thélohan, 1893) and *E. symphodi* Daoudi, Radujkovic, Marquès and Bouix, 1989 (Table 1). Only *E. catalana* resembles the species described here. *Bodianus speciosus* and *Scarus hoefleri* are new hosts.

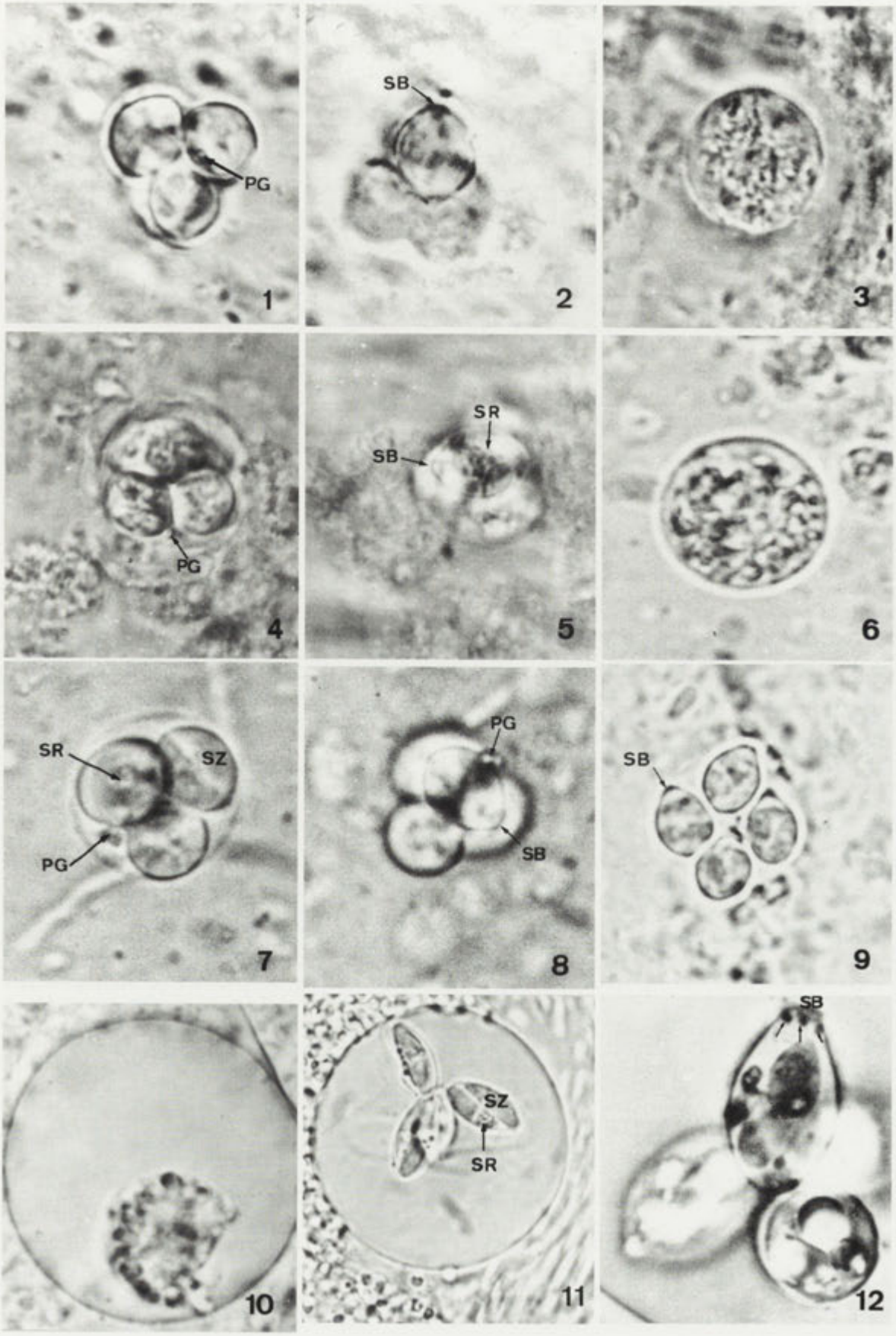
### *Eimeria dakarensis* Faye, 1988 (Figs. 9, 33, 42)

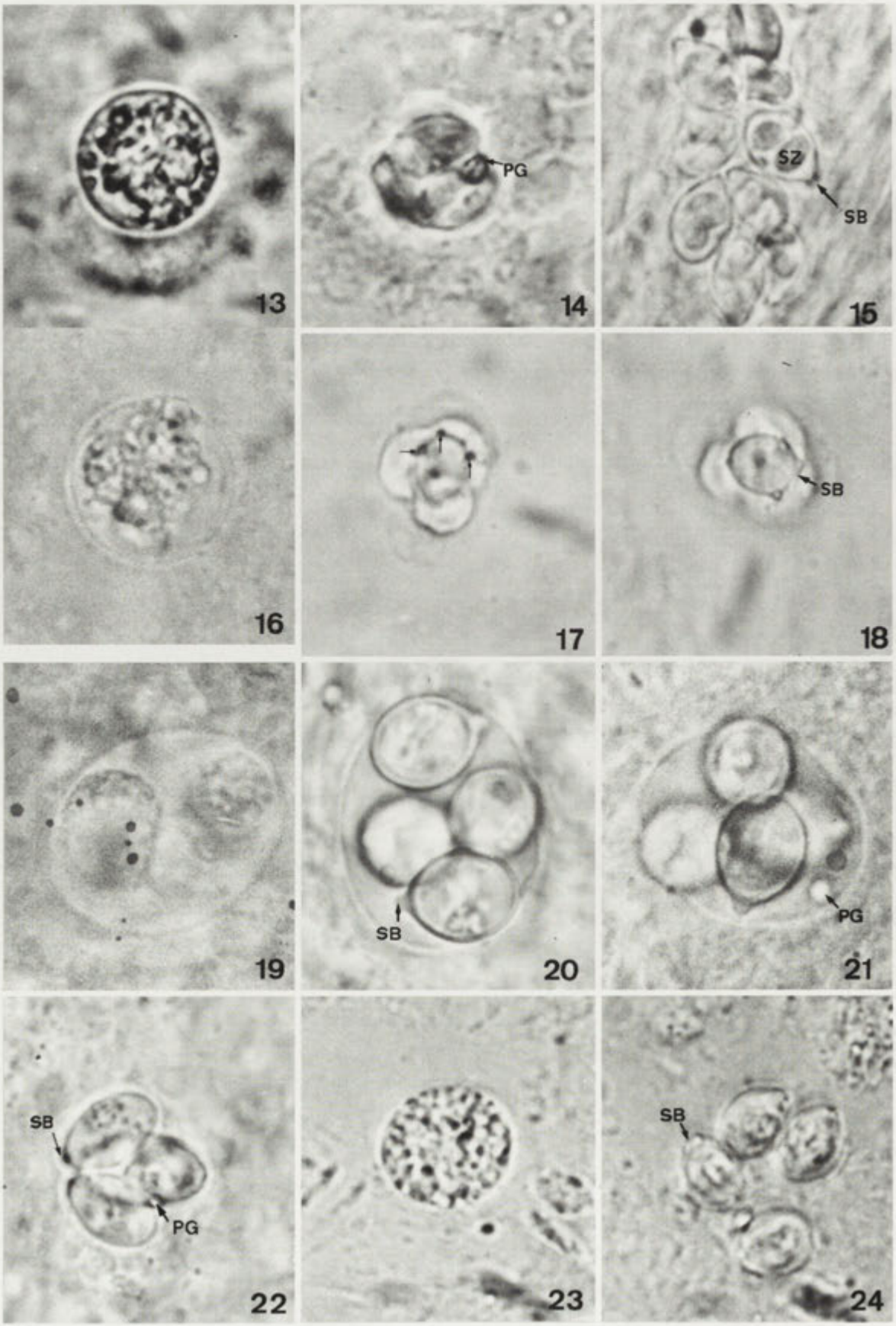
Hosts: *Cephalopholis taeniops* (Valenciennes, 1828) (Perciformes: Serranidae) and *Epinephelus alexandrinus* (Valenciennes, 1828) (Perciformes: Serranidae).

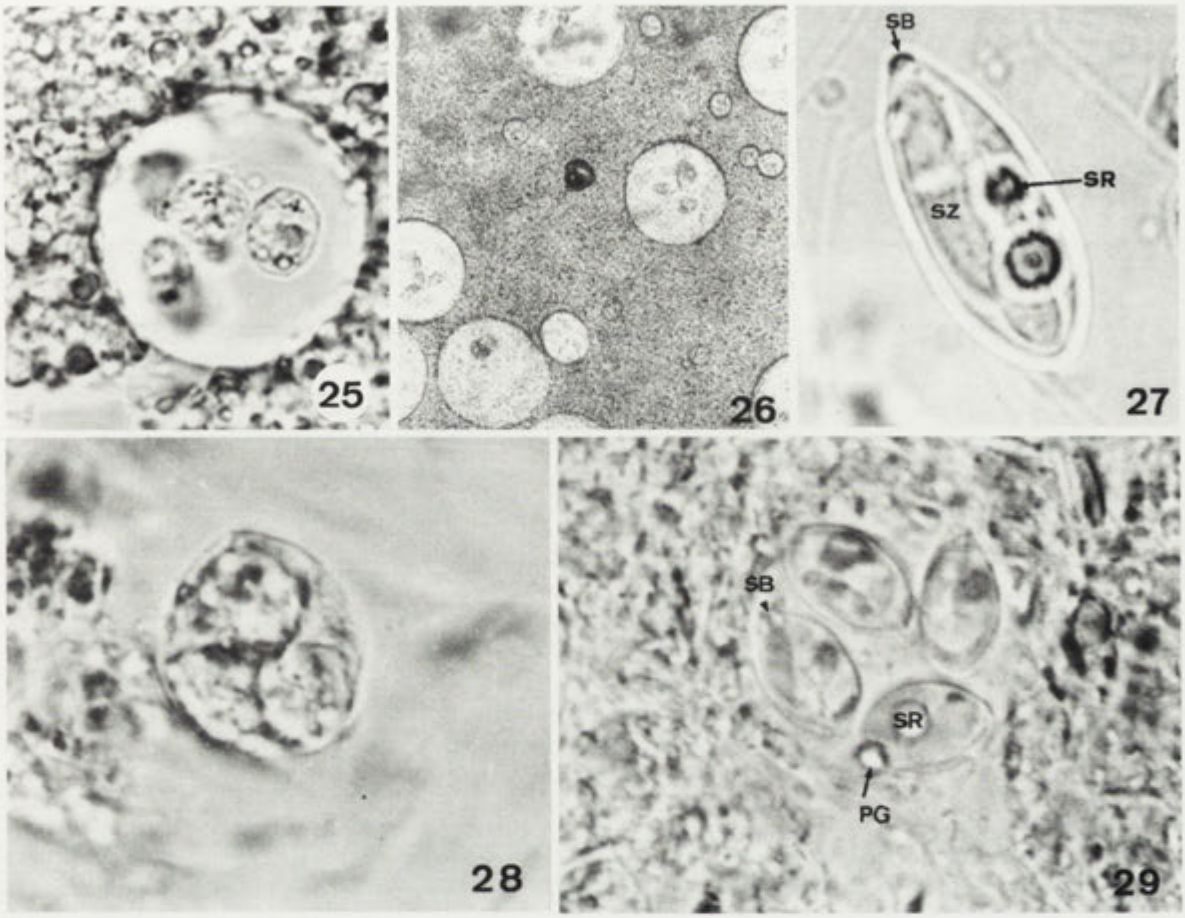
Sites of infection: intestine and pyloric caeca.

Prevalence : 24.2% (8/33) for *C. taeniops* and 14.2% (4/28) for *E. alexandrinus*.

Description: oocysts irregular in shape, often spherical and measure 11.4 ± 0.7 µm in diameter. Oocyst








Figs. 1-29. Light micrographs of *Eimeria* oocysts and zygotes. 1, 2 - *E. adioryxi*. Sporulated oocysts. PG - polar granule, SB - stieda body (x 2300). 3 - *E. ashburneri*. Oocyst containing young sporoblasts (x 2300). 4, 5 - *E. ashburneri*. Sporulated oocysts. PG - polar granule, SB - stieda body, SR - sporocyst residuum (x 2300). 6 - *E. catalana*. A zygote (x 2300). 7, 8 - *E. catalana*. Sporulated oocysts. PG - polar granule, SB - stieda body, SR - sporocyst residuum, SZ - sporozoite (x 2300). 9 - *E. dakarensis*. Sporulated oocyst. SB - stieda body (x 2300). 10 - *E. ethmalosae*. A zygote (x 2300). 11 - *E. ethmalosae*. Sporulated oocyst. SR - sporocyst residuum, SZ - sporozoite (x 1900). 12 - *E. ethmalosae* Sporocysts. SB - stieda body. Arrows show the papillae (x 2300). 13 - *E. gabonensis*. A zygote (x 2300). 14 - *E. gabonensis*. Sporulated oocyst. PG - Polar granule (x 2300). 15 - *E. gabonensis*. Sporocysts. SB - stieda body, SZ - sporozoite (x 2300). 16 - *E. ivanae*. A zygote (x 2300). 17, 18 - *E. ivanae*. Sporulated oocysts. SB - stieda body. Little arrows show polar granules (x 2300). 19 - *E. kayarensis*. Oocyst containing sporoblasts (x 2300). 20, 21 - *E. kayarensis*. Sporulated oocysts. PG - polar granule, SB- stieda body (x 2300). 22 - *E. perciformis*. Sporulated oocyst. PG - polar granule, SB - stieda body (x 2300). 23 - *E. ryptici*. A zygote (x 2300). 24 - *E. ryptici*. Sporulated oocyst. SB - stieda body (x 2300). 25 - *E. sardinae*. Oocyst containing sporoblasts (x 1900). 26 - *E. sardinae*. Sporulated oocysts of varying size (x 100). 27 - *E. sardinae*. A sporocyst. SB - stieda body, SR - sporocyst residuum, SZ - sporozoite (x 1900). 28 - *E. syacii*. Oocyst containing sporoblasts ( x 2300 ). 29 - *E. syacii*. Sporulated oocyst. PG - p:olar granule, SB - stieda body, SR - sporocyst residuum (x 2300)

residuum absent but 3 polar granules were observed (Fig. 33). Each mature oocyst contains four pear-shaped sporocysts measuring 6.8 ± 0.6 µm x 4.6 ± 0.9 µm with a Stieda body at the tapered end (Figs. 9, 33). Sporocyst residuum consists of 4 refringent granules. Each of the mature sporocysts contains two peripheral vermiform sporozoites (Fig. 33). In SEM, oocyst surface presents craters varying in size (Fig. 42).

Remarks: the oocysts observed are slightly smaller than those observed by Faye (1988) but sporocysts have the same size. *Epinephelus alexandrinus* is a new host.

***Eimeria ethmalosae* sp. n. (Figs. 10, 11, 12, 34, 43)**

Host: *Ethmalosa fimbriata* (Bowdich, 1925) (Clupeiformes: Clupeidae).

Site of infection: testes.

Prevalence: 69.2% (9/13) of males observed.

Description: oocysts spherical. In SEM (Fig. 43), the oocyst wall is thin and presents small craters. Each mature oocyst contains four ellipsoidal sporocysts. Stieda body with papillae at the tapered end of the sporocysts (Fig. 12). Sporocysts are often eccentric and occupy a little part

Table 1
*Eimeria* species of fishes belonging to the family Labridae

| Species | Characters (dimensions in µm) | Hosts and sites of infection |
|---|---|---|
| *Eimeria catalana Lom and Dykovà, 1981* | Oocysts spherical: 11 (10.5-11.5), three or six polar granules. Sporocysts ellipsoidal. 7.7 (7,5-8.5) x 6 (5.5-6.5). Stieda body ring like. Sporocyst residuum granulated. | *Crenilabrus mediterraneus* (intestine) |
| *Eimeria banyulensis* Lom and Dykovà, 1982 | Oocysts spherical: 7.8 (6.5-8.5). Sporocysts ellipsoidal: 3.5 (3-4) x 5 (4.5-5.5). Stieda body non observed. Sporocysts residuum granulated. | *Coris julis*, *Ctenolabrus rupestris*, *Symphodus mediterraneus* (intestine) |
| *Eimeria roussillona* Lom and Dykovà, 1981 | Oocysts spherical: 11 (10-12). Sporocysts ellipsoidal: 4.1 (3.0-4.5) x 7.8 (7.8-8.5). Stieda body like a flat thickening. Sporocyst residuum with few granules. | *Labrus turdus* (intestine) |
| *Eimeria variabilis* (Thélohan, 1981) | Oocysts spherical: (9.2-10.9) x (13.9-14.3). Sporocysts elongated: (8.5-9.2). Stieda body present. Sporocyst residuum absent. | *Cottus bubalis* (pyloric caeca) |
| *Eimeria petrovitchi* Daoudi, 1987 | Oocysts spherical: 12 (11- 12.5). Sporocyst residuum formed by twenty granules. | *Symphodus ocellatus* (intestine) |
| *Eimeria symphodi* Daoudi, Radujkovic, Marquès and Bouix, 1989 | Oocysts spherical: 17.3 (16-19). Sporocysts ellipsoidal: 12.1 (11-13) x 5.8 (5.5-6.3). Stieda body like a circular thickening. | *Symphodus rostratus* (intestine) |

of the oocyst volume (Fig. 11). Each of them contains two vermiform sporozoites arranged head to tail (Figs. 11, 12, 34). Sporocyst residuum consists of 4 or 5 refringent granules. Zygotes (Fig. 10) were also observed.

This coccidium presents oocysts varying in size. Three categories were observed in the same host. Small oocysts: 22 ± 0.9 µm in diameter with sporocysts measuring 9.2 ± 0.6 µm x 6.7 ± 0.4 µm; medium-sized oocysts: 31 ± 1.1 µm in diameter with sporocysts measuring 10.8 ± 0.7 µm x 7.3 ± 0.8 µm; and giant-sized oocysts: 53 ± 1.2 µm in diameter with sporocysts measuring 12.5 ± 0.6 µm x 8.3 ± 0.7 µm. These categories are in fact theoretic because intermediate sizes were observed.

Remarks: *Eimeria* species producing giant-sized species oocysts have also been reported from testes of Clupeiformes fishes. These are: *Eimeria sardinae* (Thélohan, 1890), Reichenov, 1921; *Eimeria nishin* Fujita, 1934; *Eimeria etrumei* Dogiel, 1940 and *Eimeria brevoortiana* Hardcastle, 1944 (Pellerdy, 1965; Dykovà and Lom, 1983; Upton et al. 1984). Only *E. brevoortiana* from *Brevoortia tyranus* produces sporocysts with Stieda body but the oocysts are ellipsoidal, and the sporocysts are longer. *Eimeria myoxocephali* described by Fitzgerald (1975) from intestine of *Myoxocephalus polyacanthocephalus* is similar in appearance to our species, but the Stieda body is absent. We think that the species observed here is new and we propose to call it *Eimeria ethmalosae* from the generic name of its host. This coccidium is the second species described from *Ethmalosa fimbriata*. The first, *Goussia ethmalotis*, infects the liver and have been described by Obiekezie (1986) in Nigeria.

***Eimeria gabonensis* sp. n. (Figs. 13, 14, 15, 35)**

Host: *Chelidonichthys gabonensis* (Poll and Roux, 1955) (Scorpaeniformes: Triglidae)

Site of infection: intestine.

Prevalence: 50% (2/4)

Description: oocysts spherical: 9.6 ± 0.6 µm in diameter (Fig. 35). Oocyst residuum absent but one polar granule measuring 1.7 ± 0.3 µm in diameter is present. A few zygotes with granular cytoplasm have also been observed among sporulated oocysts (Figs. 14, 35). Each mature oocyst contains four ellipsoidal sporocysts measuring 6.8 ± 0.6 µm x 4.6 ± 0.5 µm. Stieda body splitlike at the tapered end of the sporocyst (Figs. 15, 35). Sporocyst residuum consists of 4 refringent granules. Sporocyst contains two falciform sporozoites arranged in the shape of a cross (Fig. 35).

Remarks: the *Eimeria* described here resembles *E. tedlai* Molnar and Fernando, 1914 and *E. triglae*

Table 2
*Eimeria* species of fishes resembling *Eimeria gabonensis* by the size of oocysts and sporocysts

| Species | Characters (dimensions in µm) | Hosts and sites of infection |
|---|---|---|
| *Eimeria tedlai* Molnar and Fernando, 1974 | Oocysts spherical: (9.5-10). One polar granule present. Sporocysts ovoid: (8-8.4) x (4.5-4.7). Stieda body caplike. Sporocyst residuum compact. | *Perca flavescens* (intestine) |
| *Eimeria triglae* Daoudi, Radujkovic, Marquès and Bouix, 1989 | Oocysts spherical: 9.8 (9-11) x 5 (4.5-5.5). Stieda body like a protruding pass. Sporocyst residuum formed by four or six refringent granules. | *Trigla lucerna* (pyloric caeca) |
| *Eimeria gabonensis* sp. n. | Oocysts spherical: 9.6 (9-11). One polar granule present. Sporocysts ellipsoidal: 6.8 (6-8) x 4.6 (4-5.5). Stieda body with a crack in the middle. | *Chelidonichthys gabonensis* (intestine) |

Daoudi, Radujkovic, Marquès and Bouix, 1989 by the size of its oocysts and sporocysts (Table 2), but differs from these species by the shape of its sporocysts. We consider this is a new species for which we propose the name *Eimeria gabonensis*.

***Eimeria ivanae* Lom and Dykovà, 1981 (Figs. 16, 17, 18, 36)**

Hosts: *Serranus cabrilla* (Linnaeus, 1758) (Perciformes: Serranidae) and *Serranus scriba* (Linnaeus, 1758) (Perciformes: Serranidae).

Site of infection: intestine, feces.

Prevalence: 35% (14/40) for *S. cabrilla* and 18.7% (3/16) for *S. scriba*.

Description: a few zygotes with granular and often eccentric cytoplasm have been observed among sporulated oocysts (Fig. 16). Oocysts spherical, measuring 9.8 ± 0.6 µm in diameter. Oocyst residuum absent but 2 or 6 little polar granules have been observed (Figs. 17, 36). Each mature oocyst contains four ovoid sporocysts 7.3 ± 0.7 µm x 5.5 ± 0.3 µm. Stieda body consists of a circular thickening at one end of the sporocyst (Figs. 18, 36).

Remarks: this species has been described by Lom and Dykovà (1981) from *Serranus cabrilla,* but they didn't observe polar granules. Nevertheless its location and the shape of its oocysts and sporocysts prove that this is *E. ivanae*. *Serranus scriba* is a new host for this coccidium.

***Eimeria kayarensis* sp. n. (Figs. 19, 20, 21, 37, 44, 45)**

Host: *Raja miraletus* Linnaeus, 1758 (Rajiformes: Rajidae).

Site of infection: spiral valve.

Prevalence: 41.6% (5/12)

Description: oocysts ellipsoidal: 16.7 ± 1.1 µm x 13.9 ± 0.9 µm (Figs. 19, 20, 21). Oocyst wall thin and smooth (Fig. 44). Oocyst residuum absent but two polar granules present. (Fig. 21). Oocysts containing sporoblasts have been observed among sporulated oocysts (Fig. 19). They are also ellipsoidal. Stieda body knoblike (Figs. 20, 45). Sporocyst wall smooth in SEM (Fig. 45). Sporocyst residuum consists of few scattered refringent granules. Two falciform, often peripheral sporozoites can be seen in each mature sporocyst (Fig. 37).

Remarks: to our knowledge, six coccidium of the genus *Eimeria* have been described from fish belonging to the order Rajiformes. These are: *Eimeria rajarum* Van den Berghe, 1937; *E. squali* Fitzgerald, 1975; *E. quentini* Boulard, 1977; *E. ottojiroveci* Dykovà and Lom, 1983; *E. halleri* Upton, Gardner and Duszynski, 1988. Our species differs from all of them (Table 3) by the presence of polar granules and by the size of the oocysts. We consider this is a new one and propose to name it *Eimeria kayarensis* from one of the localities we have found its host. This is the second coccidium described from *Raja miraletus*. The first, *E. ottojiroveci* infects the gut epithelium.

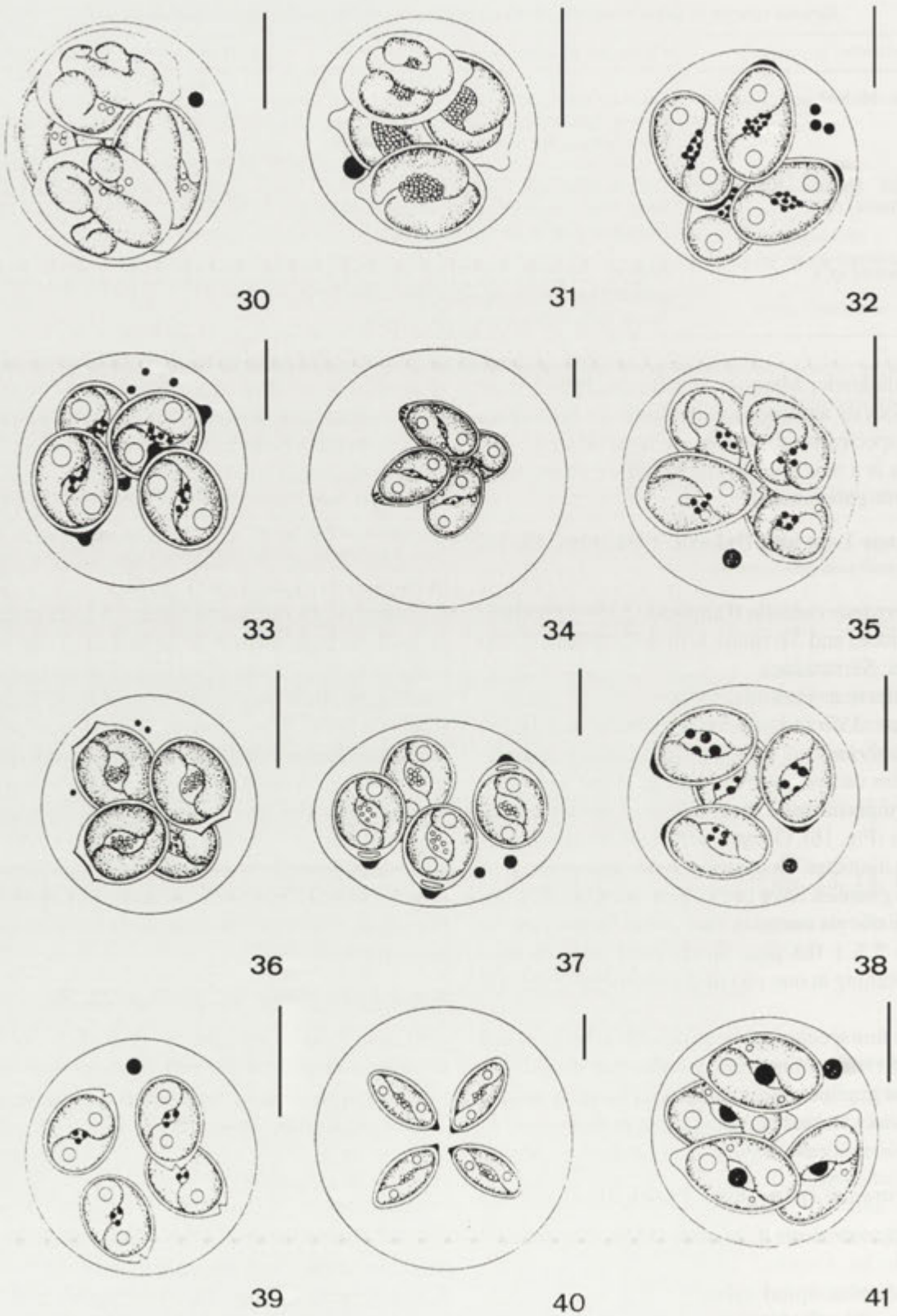
***Eimeria perciformis* sp. n. (Figs. 22, 38)**

Hosts: *Pomadasys incisus* (Bowdich, 1825) (Perciformes: Haemulidae) and *Epinephelus goreensis* (Valenciennes, 1830) (Perciformes: Serranidae).

Site of infection: intestine.

Prevalence: 5.2% (1/19) for *P. incisus* and 33.3% (1/3) for *E. goreensis*.

Description: oocysts irregular in shape, often spherical and measuring 9.7 ± 0.6 µm in diameter. Oocyst residuum absent but one polar granule 0.7 ± 0.3 µm in diameter present (Figs. 22, 38). Zygotes with granular cytoplasm have been observed among sporulated oocysts. Each mature oocyst contains four ellipsoidal sporocysts 5.9 ± 0.8 µm x 3.9 ± 0.3 µm (Fig. 38). Stieda




Figs. 30-41. Diagrammatic representation of *Eimeria* oocysts (Bar - 4 µm). 30 - E. *adioryxi*; 31 - *E. ashburneri*; 32 - *E. catalana*; 33 - *E. dakarensis*; 34 - *E. ethmalosae*; 35 - *E. gabonensis*; 36 - *E. ivanae*; 37 - *E. kayarensis*; 38 - *E. perciformis*; 39 - *E. ryptici*; 40 - *E. sardinae*; 41 - *E. syacii*

Table 3
*Eimeria* species of fishes belonging to the order of Rajiformes

| Species | Characters (dimensions in µm) | Hosts and sites of infestation |
|---|---|---|
| *Eimeria rajarum* Van den Berghe, 1937 | Oocysts weakly spherical: 22.4 x 20 (20-26.2 x 17.5-23). Polar granule absent. Sporocyst: 7 x 6.2. Sporozoites commalike. | *Raja batis* (intestine) |
| *Eimeria squali* Fitzgerald. 1975 | Oocysts ovoid to ellipsoidal: 20-29 x 24-36. Polar granule absent. Sporocysts: 5.9 x 19.6. Sporozoites at the posterior end. | *Squalus acanthias* (spiral valve) |
| *Eimeria quentini* Boulard, 1977 | Oocysts elongated: 37.8 x 12.6 (22-45.9 x 10.7-15.3). Polar granule absent. Sporocyst piriform: 11.2 x 83 (9.3-12.4 x 6.2-9.3) | *Aetobatis narinari* (peritoneum) |
| *Eimeria ottojiroveci* (Lom and Dykovà, 1981) Dykovà and Lom, 1983 | Oocysts spherical to ovoid: 8.3 x 6.3 (8-8.5 x 6-6.5). Sporozoites vermiform with one end wider than the other. | *Raja clavata, Raja miraletus* (intestine, spiral valve) |
| *Eimeria halleri* Upton, Bristol, Gardner and Duszynski, 1986 | Oocysts spherical to subspherical: 16.9 x 16.8 (15-18 x 15-16). Polar granule absent. Sporocysts ovoid: 11.1 x 6-7.5. Substieda body present. Sporozoits commalike. | *Urolophus halleri* (spiral valve) |
| *Eimeria chollaensis* Upton, Gardner and Duszynski, 1988 | Oocysts ovoid: 13.3 x 9.7 (11.2-16 x 8-10.8). Polar granule absent. Sporocysts ovoid: 8.9 x 4.9 (8-10 x 4.2-5.6). Sporozoites elongated with ten striations at the anterior end. | *Urolophus halleri* (spiral valve) |
| *Eimeria kayarensis* sp. n. | Oocysts ellipsoidal: 16.7 x 13.9 (14.5-18.5 x 12-15.5). Two polar granules present. Sporocysts ovoid: 7.9 x 6 (6-9 x 4.4-7). Substieda body present. Sporozoites peripheral, falciform, with one end wider than the other. | *Raja miraletus* (spiral valve) |

body is a thickening of the end of the sporocyst (Figs. 22, 38). Sporocyst residuum consists of four refringent granules between two peripheral and falciform sporozoites in each mature sporocysts.

Remarks: Faye (1988) has described *Eimeria* sp. from the intestine of *Pomadasys incisus*. It differs from the species we describe here because it has larger oocysts (14.58 µm - 15.39 µm in diameter ) and sporocysts (7.1 - 8. 5 µm x 4.8 - 6 µm). Compared to other fish coccidia, only *Eimeria pleurostici* resembles our species by the size and the shape of its oocysts and sporocysts. Nevertheless it differs from it by the form of the Stieda body (Molnar and Rohde 1988a). We consider it a new species and we propose the name *Eimeria perciformis,* from the order to which belong its hosts.

### *Eimeria ryptici* sp. n. (Figs. 23, 24, 39)

Host: *Rypticus subbifrenatus* Gill, 1861 (Perciformes: Grammistidae)

Site of infection: intestine.

Prevalence: 50% (1/2)

Description: a few zygotes (Fig. 23) with granular cytoplasm have been observed among sporulated oocysts. Oocysts spherical, 8.1 ± 0.5 µm in diameter. Oocyst residuum absent but one polar granule measuring 1.4 ± 0.2 µm x 0.9 ± 0.1 µm is present (Fig. 39). Each mature oocyst contains four ovoid sporocysts 4.9 ± 0.3 µm x 3.4 ± 0.4 µm large. Stieda body splitlike (Figs 24, 39). Three or four refringent granules form the sporocyst residuum. Each mature sporocyst contains two falciform and peripheral sporozoites (Fig. 39).

Remarks: this species resembles *Eimeria gabonensis* (in this work) and *Eimeria triglae* Daoudi, Radujkovic, Marquès and Bouix, 1989 (Table 4). But the hexagonal shape of the spozocysts of *E. triglae* and the big size of oocysts and sporocysts in *E. gabonensis* differ these species from the coccidia described here. We consider it a new species and we propose to name it *Eimeria ryptici* from the generic name of its host.

Table 4
*Eimeria* species of fishes resembling *E. ryptici*

| Species | Characters (dimensions in µm) | Hosts and sites infestation |
|---|---|---|
| *Eimeria triglae* Daoudi, Radujkovic, Marquès and Bouix, 1989 | Oocysts spherical: 9. Sporocysts ellipsoidal: 7.3 x 5. Sporocyst residuum formed by four or six granules.Stieda body like a protruding pass. | *Trigla lucerna* (pyloric caeca) |
| *Eimeria gabonensis* sp.n. | Oocysts spherical: 9.6. One polar granule present. Sporocysts ellipsoidal: 6.8 x 4.6. Sporocyst residuum formed by four granules. Stieda body with a crack in the middle. | *Chelidonichthys gabonensis* (intestine) |
| *Eimeria ryptici* sp. n. | Oocysts spherical: 8.1. One polar granule present. Sporocysts ovoid: 4.9 x 3.4. Sporocyst residuum in the form of three or four granules. Stieda body with a crack in the middle. | *Rypticus subbifrenatus* (intestine) |

### *Eimeria sardinae* (Thélohan, 1890) Reichenow, 1921 (Figs 25, 26, 27, 40, 46, 47)

Hosts: *Sardinella aurita* Valenciennes, 1847 (Clupeiformes: Clupeidae) and *Sardinella maderensis* (Lowe, 1839) (Clupeiformes: Clupeidae).

Site of infection: testes.

Prevalence: 50% (725/1450) of males for *S. aurita* and 65% (780/1200) of males for *S. maderensis*.

Description: this coccidium produces oocysts varying in size (Fig. 26). Small-sized oocysts have been observed from 45% of infected *S. maderensis*. They measure 10.5 ± 0.8 µm in diameter with sporocysts measuring 7.7 ± 0.7 µm x 3.7 ± 0.5 µm. Medium-sized oocysts from 30% of infected *S. maderensis* and 90% of infected *S. aurita*. Their mean diameter 21.9 ± 0.9 µm. They contain sporocysts measuring 14.1 ± 0.8 µm x 6.8 ± 0.3 µm. Giantsized oocysts from 25% of parasitized *S. maderensis* and 10% of infected *S. aurita*; their diameter is 38.9 ± 1.1 µm. They contain sporocysts measuring 17.5 ± 0.8 µm x 7.8 ± 0.6 µm. These three categories are often observed in the same host (Fig. 26). A few oocysts containing ovoid sporoblasts have also been observed among sporulated oocysts (Fig. 25). Oocysts of each category are spherical. Their wall is thin and their surface smooth (Fig. 46). Polar granule absent. They contain four fusiform sporocysts occupying a little part of the oocyst (Figs. 26, 40). Their surface is generally smooth. No suture has been observed but Stieda body is present in giant-sized sporocysts from *S. aurita* (Fig. 47). Each sporocyst contains two vermiform sporozoites rolling up lightly each another (Figs. 27, 40). About ten refringent granules form the sporocyst residuum.

Remarks: *Eimeria sardinae* is a cosmopolitan species parasitizing the testes of clupeiformes fishes. It has been found in all the oceans. *S. maderensis* is a new host for this species.

### *Eimeria syacii* n. sp. (Figs 28, 29, 41)

Host: *Syacium micrurum* Ranzani, 1840 (Pleuronectiformes: Bothidae).

Site of infection: intestine.

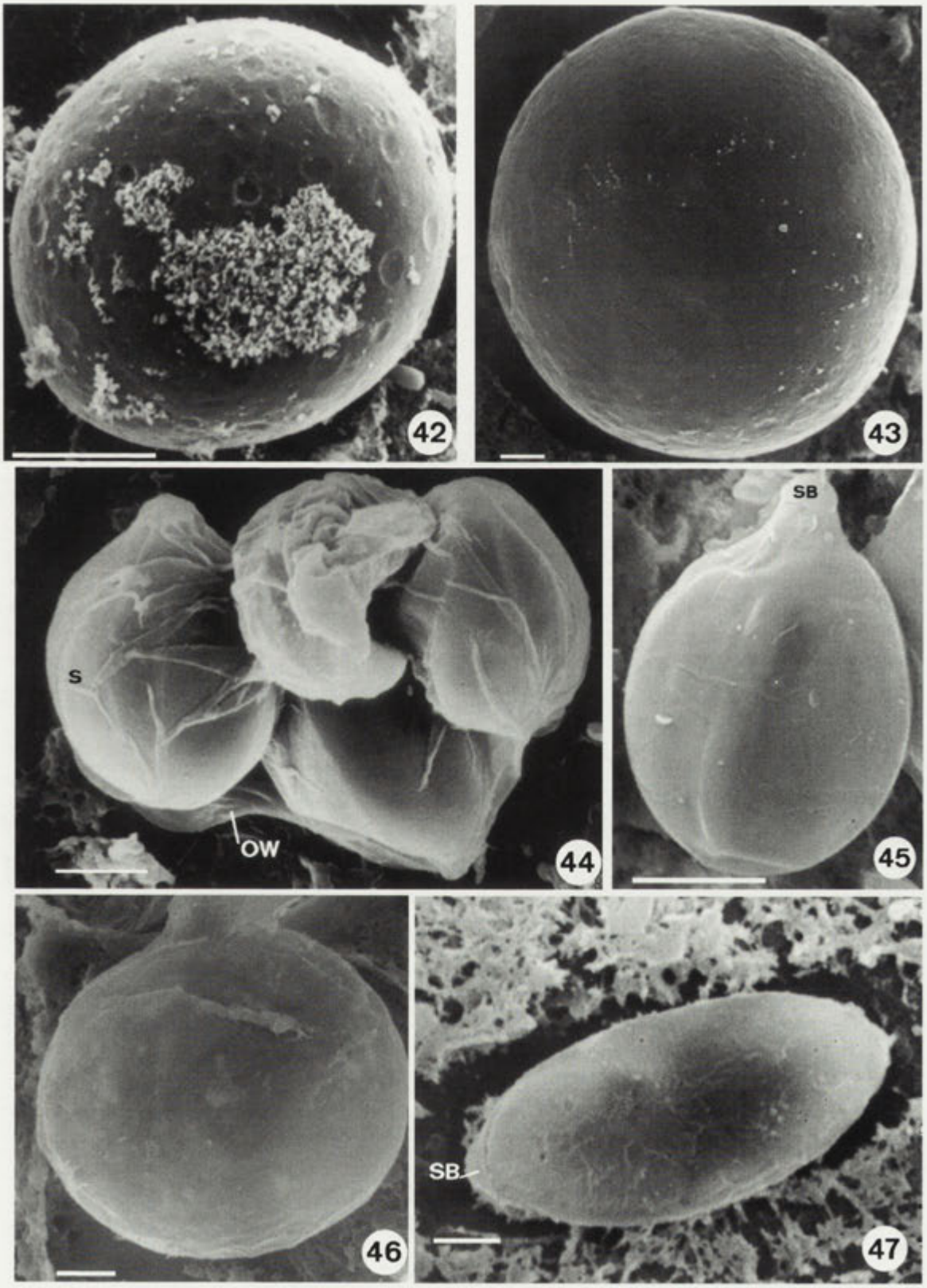
Prevalence: 16.6% (4/24)

Description: a few oocysts containing sporoblasts have been observed among sporulated oocysts (Fig. 28). Oocysts spherical (Figs. 29, 41). Diameter, 10.7 ± 1.1 µm. One polar granule measuring 0.8 ± 0.2 µm is present. Oocyst residuum absent. Each mature oocyst contains four ellipsoidal sporocysts measuring 6.6 ± 0.7 µm x 4.1 ± 0.5 µm (Figs. 29, 41). Two falciform and peripheral sporozoites surrounding a compact sporocyst residuum measuring 1.7 ± 0.3 µm (Fig. 29) are presents within each sporocyst. Stieda body is knoblike (Figs. 29, 41).

Remarks: to our knowledge only *Eimeria tedlai* Molnar and Fernando, 1974 is similar to the species described here by the size of its oocysts and sporocysts, by the presence of one polar granule and a compact sporocyst residuum. But the Stieda body of this species is not prominent (Molnar and Fernando, 1974). We consider this a new species for which we propose the name *Eimeria syacii*.

## CONCLUSION

The most important criteria for assigning coccidia to their appropriate genera are sporocysts and oocysts morphology, size and structure. We have applied those criteria to identify our coccidia. Members of the genus *Eimeria* produce oocysts with four sporocysts each with




Figs. 42-47. SEM micrographs of *Eimeria* oocysts (Bar - 4 µm). 42 - *E. dakarensis*. Oocyst. 43 - *E. ethmalosae*. Oocyst. 44 - *E. kayarensis*. Sporulated oocyst. OW - oocyst wall, S - sporocyst. 45 - *E. kayarensis*. Sporocyst. SB - stieda body. 46 - *E. sardinae*. Oocyst. 47 - *E. sardinae*. Sporocyst. SB - stieda body

two sporozoites. Stieda body is present at one of the ends of the sporocysts. All the species described here present these characters.

*Eimeria sardinae* and *Eimeria ethmalosae* found in fishes from senegalese coasts produce three types of oocysts: micro-oocysts, meso-oocysts and macro-oocysts. We have no explanation for this phenomenon. About host specificity two cases have been observed: a less strict host specificity, that is the case of *Eimeria perciformis* parasitizing a Haemulidae (*Pomadasys incisus*) and a Serranidae (*Epinephelus alexandrinus*) and a strict host specificity as the case of *Eimeria ethmalosae* described from the testis of *Ethmalosa fimbriata*.

All the *Eimeria* found are histozoic and can be pathogenic for their hosts. *Eimeria kayarensis* destroys the intestinal epithelium of *Raja miraletus* and *E. sardinae* induces the atrophy of the testes of *Sardinella maderensis* (Diouf and Toguebaye 1994). Pinto (1956) affirmed that *Eimeria sardinae* occupies the whole of the testis in *Sardina pilchardus*, thus causing a veritable parasitic castration.

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# New Myxosporeans (Myxozoa: Myxosporea) from Marine Fishes of Tasmania, Australia

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**Summary.** Six new species of Myxosporea were recorded from marine fishes collected at south-eastern Tasmania, Australia. *Sphaeromyxa nesogobii* sp. n. from the gall bladder of *Nesogobius* sp.1; *Zschokkella macrocapsula* sp. n. from the gall bladder of *Atherinosoma microstoma* and *Leptatherina presbyteroides*; *Ortholinea striateculus* sp. n. from the ureta of *Leptatherina presbyteroides*; *Ceratomyxa arripica* sp. n. from the gall bladder of *Arripis trutta*; *Sphaerospora aldrichettae* sp. n. from the gall bladder and *Myxobolus aldrichetti* sp. n. from the gills of *Aldrichetta forsteri.*

**Key words.** Myxosporea, new species: *Sphaeromyxa nesogobii, Zschokkella macrocapsula, Ortholinea striateculus, Ceratomyxa arripica, Sphaerospora aldrichettae, Myxobolus aldrichetti*, fish parasite.

## INTRODUCTION

Studies on fish-infecting protozoans in Australia are very few, especially given the large number of fish species which are found in the country. Since the first paper dealing with the myxosporean parasites was published (Johnston and Cleland 1910), only 25 species of Myxosporea have been reported from Australian fishes; 16 of these were from marine fishes (Johnston and Bancroft 1918; Woolcock 1936; Willis 1949; Blackburn 1960; Lester 1979, 1982; Langdon 1987, 1990, 1991; Moser et al. 1989; Kent and Moser 1990; Lom et al. 1992). During a survey for protozoan parasites of Tasmanian marine fishes, seven myxosporean species were found. These species are morphologically different from the hitherto known species. Six of these are described in the present paper. An account of the other species, *Zschokkella leptatherinae* has already been submitted for publication (Su and White, in press).

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## MATERIALS AND METHODS

One thousand three hundred and eighty five coastal fishes belonging to 12 species were collected from the seagrass meadows at Dru Point, North-West Bay, in south-eastern Tasmania, Australia from January 1990 to June 1992. Robertson and White (1986) gave a full description of the site and the collecting methods. The detection of myxosporean parasites started with searching for cyst-like structures. Fishes were then killed and dissected immediately. Myxosporean spores were examined from fresh preparations of cyst or from the tissue pieces of liver, spleen, gall bladder, kidney, ureta, swimbladder and brain with a Zeiss Standard Universal microscope equipped with phase contrast and 1600x oil immersion lens. Temporary preparations of myxosporean spores stained with Lugol's solution were used in order to detect the iodinophilous vacuole. Stained preparations were made by Giemsa method.

All measurements were made on 50 fresh specimens and given in micrometers (μm) unless indicated. Minimum and maximum values were given, followed in parentheses by the arithmetic mean. Figures were drawn with the aid of a camera lucida. Photomicrographs were taken using a low speed ILFORD FP4 film. Holotype slides are deposited in the Tasmanian Museum and Art Gallery (TMAG), Argyle Street, Hobart, Tasmania, Australia 7000.

## DESCRIPTIONS

### *Sphaeromyxa nesogobii* sp. n. (Figs. 1A; 3A, B)

Host: *Nesogobius* sp.1 (name as given in Last et al., 1983)

Location in host: Gall bladder

Date: October 1990

Type material: Holotype TMAG-K1301

Prevalence of infection: Out of 23 fish specimens examined, only one was found to contain several hundreds of spores.

Vegetative form: Not found

Spores: The spores are elongate fusiform, arcuate in both valvular and sutural views. The ends of the spores are bluntly rounded to truncated, slightly curving in two directions. In valvular view, the spores are concave on one side and convex on the other. They are 20.5-23.7 (21.7) μm in length and 6.3-7.9 (6.6) μm in width. The spore valves are smooth, without striation. The sutural ridge is slightly curved. The two polar capsules are situated in the opposite poles of the spore. They are pyriform, 6.8-7.9 (7.6) μm in length and 3.1-3.3 (3.2) μm in width. The polar filaments fold 3-5 times parallel to the long axis of the spore within the polar capsules. The sporoplasm is situated in the space between the two polar capsules, two nuclei are visible amongst it. The capsular nuclei are often visible beneath the polar capsules.

#### Remarks

Laird (1953) divided the genus *Sphaeromyxa* into two groups. The *balbianii* group is characterized by straight or slightly curved fusiform or ovoid spores and ovoid polar capsules, while the *incurvata* group has the arcuate spores with pyriform polar capsules. The present species is a member of the *incurvata* group. Comparing the present myxosporean with other previously recorded species, *Sphaeromyxa nesogobii* resembles *S. parva* Dogiel, 1948 (Schulman 1966) from *Cololabis saira* and *Pholis pictus*; *S. hellandi* Auerbach, 1909 (Schulman 1966) from *Phollis gunellus*, *Brosme brosme*, *Molva molva;* and *S. elegini* Dogiel, 1948 (Schulman 1966) from *Eleginus gracilis* in having smooth spore valves and a slightly arcuate spore. However, *S. parva* and *S. elegini* are markedly different from the present species in having smaller spores and polar capsules. Although the dimensions of the spores and polar capsules of the present species are close to those of *S. hellandi*, the two species can be distinguished by two features: the spores of *S. hellandi* curve in one direction, while the spores of *S. nesgobii* curve in two different directions; the distance between the two polar capsules in *S. hellandi* is comparatively small and usually does not exceed the length of polar capsules, while in *S. nesogobii*, it is generally longer and occasionally, it is slightly smaller than the polar capsules. The measurements of the present species and three previously reported species are given in Table 1. In view of these morphological differences, geographical distribution as well as the host species; we designate the present species new and the name is given to refer to the host fish.

### *Zschokkella macrocapsula* sp. n. (Figs. 1B; 4A, B)

Hosts: *Leptatherina presbyteroides* and *Atherinosoma microstoma*

Location in host: Gall bladder

Date: August, September, November and December 1990

Type material: Holotype TMAG-K1302

Prevalence of infection: Three of 589 *Leptatherina presbyteroides* and two of 514 *Atherinosoma microstoma* contained a few spores.

Vegetative form: Not found

Table 1

The biometrical features of *Sphaeromyxa nesogobii* sp. n., *S. parva*, *S. hellandi* and *S. elegini* (minimum and maximum values were followed by the arithmetic mean in parentheses)

| | *S. nesogobii* | *S. parva* | *S. hellandi* | *S. elegini* |
|---|---|---|---|---|
| Length of spore | 20.5-23.7 (21.7) | 15.0-17.0 | 20.5-23.0 | 17.0-20.0 |
| Width of spore | 6.3-7.9 (6.6) | 4.0 | 3.5-5.0 | 6.0 |
| Length of polar capsule | 6.8-7.9 (7.6) | 5.0 | 7.0-8.5 | 5.0-6.0 |
| Width of polar capsule | 3.1-3.3 (3.2) | ### | ### | ### |

### no information.

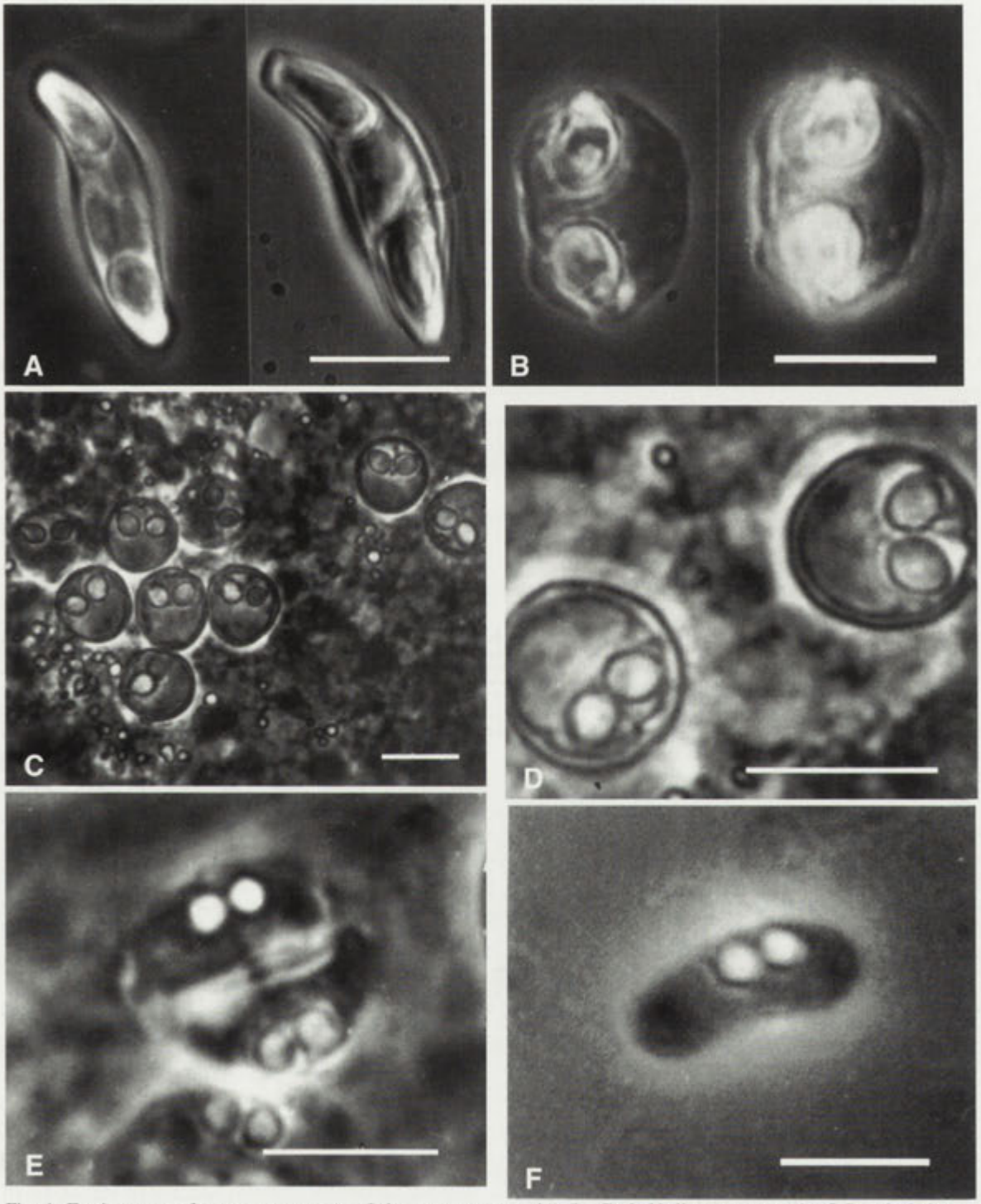


Fig. 1. Fresh spores of myxosporeans. A - *Sphaeromyxa nesogobii*, B - *Zschokkella macrocapsula*, C-D - *Ortholinea striateculus*, E-F - *Ceratomyxa arripica*. Bar - 10 µm

Spores: The spores are ellipsoidal or ovoid in both valvular and sutural views. The sutural ridge runs from one pole of the spore to one side before reaching the other pole. The spore valves are smooth and thicken. Measurements were made on 20 fresh spores. The spores are 14.0-17.5 (16.4) µm long and 12.6-14.6 (12.9) µm wide. Two polar capsules are ovoid or pyriform in shape and open near the poles of spore. They are relatively large and both are equal in size, measuring 6.3-8.4 (6.9) µm in length and 4.3-6.2 (5.1) µm in width. The polar

filaments coil 8-10 times. The sporoplasm is situated between the two polar capsules. Two nuclei are present among homogeneous sporoplasm; the diameter of nuclei is c. 1.4 μm.

Remarks

The overall morphological characters of spores of the present species resemble those of *Z. embiotocidis* Moser and Haldorson, 1976 from the gall bladder of *Damalichthys vacca* and *Embiotoca lateralis* and *Z. rovigenesis* Nemeczek, 1922 from the urinary bladder of *Scorpaena scrofa* and *S. porcus*. However, *Z. macrocapsula* differs from those two species in the size of the polar capsule and the number of coils of the polar filament. Both *Z. embiotocidis* and *Z. rovigenesis* have smaller polar capsules and fewer coils in the polar filament than *Z. macrocapsula*. The measurements of spores of *Z. macrocapsula* most closely resemble those of *Z. meglitschi* Moser and Noble, 1976 in macrourid fish. However, the shape in sutural view of the two species is distinctly different and the dimensions of the polar capsule are greater in the present species. Moreover, the polar filament in *Z. meglitschi* has 6-8 coils. In view of these morphological differences and geographical distribution, we consider the species described here to be new. The proposed trivial name refers to the size of the polar capsule.

***Ortholinea striateculus* sp. n. (Figs. 1C, D; 5A, B)**

Host: *Leptatherina presbyteroides*
Location in host: Ureta
Date: November 1990
Type material: Holotype TMAG-K1303
Prevalence of infection: Only two of 589 fish specimens examined were infected with a few spores.
Vegetative form: Not found

Spores: The spores are subspherical in valvular view and oval in sutural view. Measurements were made on 20 fresh spores. The size of the spores range from 9.1 to 10.5 (10.1) μm in length and 8.9 to 10.4 (10.0) μm in width. There are 18-20 striations on the surface of the spore valves. A conspicuous triangular intercapsular process is visible at the anterior end of the spore. The sutural ridge is straight. The two polar capsules are equal in size and pyriform in shape, with proximal ends close to each other but the openings are widely separated from each other at the anterior end of the spores. The polar capsules are 3.4-3.6 (3.5) μm long and 2.8-3.1 (2.9) μm wide. The polar filaments coil 5-7 times. The sporoplasm occupies almost all the extra-capsular space, just below the polar capsules. Two sporoplasm nuclei are visible even in the fresh specimens.

Remarks

The genus *Ortholinea* was established by Schulman in 1962. To date, only eight species have been reported: *O. polymorpha* Davis, 1917 (Lom and Dyková 1992) from the urinary bladder of *Opsanus tau* and *O. beta*; *O. divergens* Thélohan, 1895 (Schulman 1966) from the uriniferous tubules of kidney and urinary bladder of *Hippoglossoides platessoides limandoides*, *Blennius folis*, *Crenilabrus melops*, *C. pava* and *Pleuronichthus verticalis*; *O. orientalis* Schulman and Schulman-Albova, 1953 (Schulman 1966) from the urinary bladder and gall bladder of *Clupea harengus pallasi*, *C. harengus pallasi n. maris-albi*, *Eleginus navaga* and *E. gracili*; *O. irregularis* Kabata, 1962 from the urinary bladder of *Drepanopsetta platessoides*; *O. gobiusi* Naidenova, 1968 from the urinary bladder of *Gobius ophiocephalus* (Lom and Dyková 1992); *O. undulans* Meglitsch, 1970 from the urinary bladder and ureters of *Caulopsetta scapha* and *Peltorhamplus novaezelandiae*; *O. alata* Kent and Moser, 1990 from the lumens of the renal tubules and collecting ducts of *Chaetodon rainfordi*; *O. australis* Lom et al., 1992 from the hepatic ducts and gall bladder of *Acanthopagrus australis* and *Rhabdosargus sarba*.

The present species differs markedly from Australian species, *O. alata* in the absence of wing-like projections at the posterior end of the spore, the latter may belong to a different order according to Lom and Dyková (1992). It can also be distinguished from another Australian species, *O. australis* in having a triangular intercapsular process and the arrangement of polar capsules. The number of striations on the surface of spore valves, the coil of polar filaments as well as the location of *O. striateculus* and *O. australis* are also different. The spores of *O. orientalis* and *O. gobiusi* can be distinguished from the present species in the shape of spores, which taper to a sharp point at their posterior ends and *O. irregularis* differs from the present species in having irregular, pyriform spores. The absence of intercapsular process, different hosts and geographical distribution separate the present species also from *O. polymorpha* and *O. divergens*. The spores of *O. dundulans* differ in the same way and also the sutural ridge is undulant in this species. Based on these differences, we consider

*Ortholinea striateculus* to be new. The name refers to the characteristics of spore valves.

***Ceratomyxa arripica* sp. n. (Fig. 1E, F; 6A, B)**

Host: *Arripis trutta*
Location in host: Gall bladder
Date: January 1991 and March 1992
Type material: Holotype TMAG-K1304
Prevalence of infection: Out of eight fish specimens examined, two were infected with several tens of spores.
Vegetative form: The plasmodia are typically rounded or ovoid with one or two elongated pseudopodia. The plasmodia are 8.3-12.6 (10.3) µm long and 5.0-7.9 (6.3) µm wide. The length of pseudopodia is 8.2-25.3 (15.7) µm. Two spores are often seen lying side by side within one plasmodium.

Spores: The spores are crescent-shaped, small and symmetrical. In sutural view, the anterior margin is arched and the posterior margin is usually nearly straight, with a very slight concavity which is sometimes more evident near the suture. The spore valves are thin and delicate; the ends are rounded and direct laterally. A straight suture line runs through the middle of the spore. Measurements were made on 20 fresh spores. The length of the spore is 4.8-5.1 (4.9) µm and the width is 9.3-11.3 (10.2) µm, more than twice the length. The two polar capsules are equal and spherical or subspherical. They are located close to each other in the anterior part of the spore next to the suture line and are 1.8-2.0 (1.9) µm in diameter. The polar filaments show 3-4 indistinct coils within the polar capsules. The length of extruded filaments is 31.1-50.6 (44.6) µm. Two sporoplasm nuclei and two capsular nuclei are visible.

Remarks

Two *Ceratomyxa* species have been previously reported from Australia: *Ceratomyxa sprenti* Moser et al., 1989 and *C. rohdei* Moser et al., 1989. Both species were found in the gall bladder of fishes from Heron Island, Queensland. The species described here is readily distinguished from those two species in the size and shape of spores. Comparing the present species with other described species elsewhere, there are four which show close resemblance to the present species in general features of the spores: *C. americana* Wierzbicka, 1987 from the gall bladder of *Scomber scomber; C. inconstans,* Jameson 1929 from the gall bladder of *Scomber japonica, Usacaranx lutescens, Trachurus novae-zelandiae* and *Helicolenus percoides; C. faba* Meglitsch, 1960 from the gall bladder of *Caulopsetta scapha* and *C. gobioidesi* Chakravarty, 1939 from the gall bladder of *Odantoamplyopus rubicundus*. However, the spores of *C. americana* differ greatly from the present described species in having sharpened spore ends and they direct downward. *C. inconstans* can be distinguished from the present species in having subequal or unequal spore valves and the posterior margin varies from convex to very concave. Although the spores of *C. fabe* and *C. gobioidesi* resemble, to some extent, those of *C. arripica,* they are distinguished from the present species in having larger spores and polar capsules. Moreover, the spore valves of *C. fabe* generally terminate in extremely broad ends. Based on these morphological differences and the host records, the present species is considered distinct and designated as *Ceratomyxa arripica* following the name of host fish.

***Sphaerospora aldrichettae* sp. n. (Fig. 7A, B)**

Host: *Aldrichetta forsteri*
Location in host: Gall bladder
Date: February 1991 and May 1992
Type material: Holotype TMAG-K1305
Prevalence of infection: Three of 11 fish specimens examined were infected with a few spores.
Vegetative form: Not found

Spores: The spores are spherical or subspherical in both valvular and sutural views. The spore valves are thin, smooth and equal in size. Measurements were made on 20 fresh spores. The spores are 6.3-7.0 (6.7) µm long and 6.2-7.0 (6.6) µm wide. The sutural ridge is straight. The two polar capsules are situated at one pole of the spores. They are equal in size and pyriform in shape, and are 2.1-2.8 (2.5) µm long and 1.5-1.8 (1.6) µm wide. Usually 4 coils of the polar filaments can be seen inside the polar capsules. Two capsulogenous nuclei are situated below the polar capsules. The sporoplasm is underneath the two polar capsules; two small compact nuclei are visible within the sporoplasm.

Remarks

To date, 48 *Sphaerospora* species have been reported from fish (Lom and Dyková 1992). Most of these are coelozoic in the urinary system. Some are histozoic and parasitize a variety of tissues; others are coelozoic in the gall bladder (Arthur and Lom 1985, Supamattaya et al.

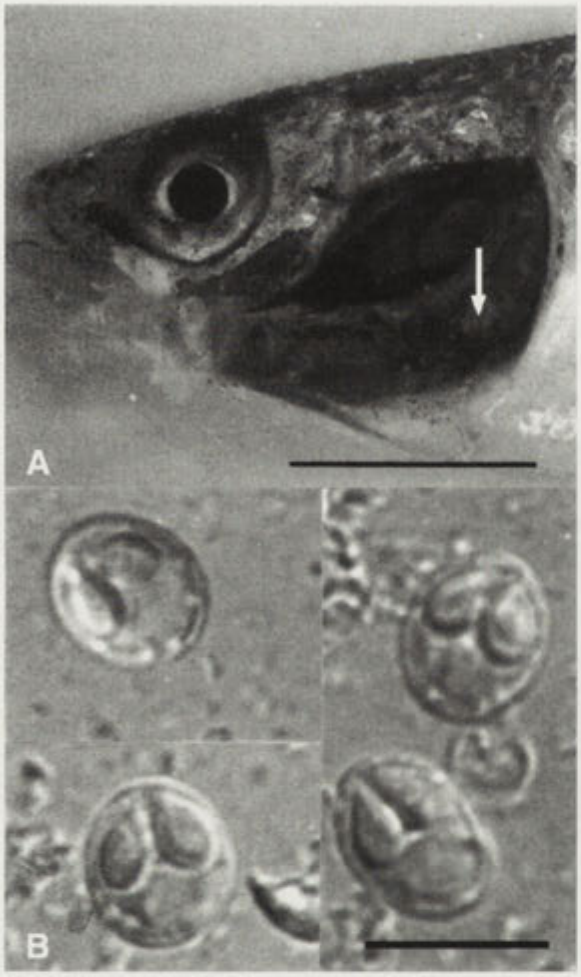


Fig. 2. *Myxobolus aldrichetti*. A - cysts (arrow) on the gills of *Aldrichetta forsteri*. Bar - 1 cm, B - fresh spores. Bar - 10 µm

1991). Nine *Sphaerospora* species were described from marine or estuarine fishes. The species described here is distinguished from *S. elegans* Thélohan, 1892 in *Gasterosteus aculeatus* (Schulman 1966); *S. renalis* Bond, 1938 in *Fundulus heteroclitu*; *S. sphaerica* Dogiel, 1948 in *Sphaeroides pardale* (Schulman 1966); *S. brevis* Polyansky, 1955 in *Myoxocephalus scorpiu* (Schulman 1966), *S. araii* Arthur and Lom, 1985 in *Raja rhina; S. epinepheli* Supamattaya et al., 1991 in *Epinephelus malabaricus* and *S. testicularis* Sitja-Bobadilla and Alvarez-Pellitero, 1990 in *Dicentrarchus labrax* by the small size of spores, site of infection and geographical distribution. Two species have been reported previously from the gall bladder of marine fishes. But both are morphologically different from *S. aldrichettae*. The spores of *S. periophami* Fantham and Porter, 1943 from *Periophthalmus koelreuteri* and *Boleophthalmus dentatus* are larger and those of *S. mayi* Moser et al., 1989 from *Atherinomous capricornensis* are smaller than new species. The sutural ridge is central in *S. aldrichettae*, but not in *S. mayi*. Furthermore, most species of *Sphaerospora* appear to be highly host specific and are known only from a single host species (Arthur and Lom 1985). Based on the above reasons, we consider *S. aldrichettae* to be new. The trivial name refers to the host fish.

***Myxobolus aldrichetti* sp. nov. (Figs. 2A, B; 8A-D)**

Host: *Aldrichetta forsteri*
Location in host: Gills
Date: February 1991
Type material: Holotype TMAG-K1306
Prevalence of infection: Out of 11 fish specimens examined, only one was infected.

Cysts: The cysts appear as ellipsoidal, opaque whitish pustules in the gill tissue. There are 8-21 cysts per infected gill arch and up to 55 cysts in the infected fish. The diameter of cysts ranges from 850 µm to 1 mm. Cysts often protrude from the surface of fish gills, and can be detected with the naked eye. Under the microscope, thousands of spores can be seen in each cyst.

Spores: The spores are regular in shape; they are ellipsoidal or ovoid in valvular view and broad lenticular in sutural and apical views. The length of the spores is 8.4-10.5 (9.7) µm and the width is 7.0-8.4 (7.7) µm. The spore valves are symmetrical and uniform in thickness, with 4-5 triangular folds on the posterior margin. The sutural ridge is distinct, broad, uniform and straight. The intercapsular process is absent. The two polar capsules are elongated pyriform and occupy at least the anterior half of the length of the spores. The polar capsules are of equal size, with dimensions of 4.2-5.2 (4.7) µm in length and 2.8-3.0 (2.8) µm in width. The polar filaments usually coil 6-7 times and remain in a tight and regular fashion inside the polar capsules. Two rod-shaped compact capsulogenous nuclei are situated below the two polar capsules. The sporoplasm occupies the whole space behind the polar capsules. Two spherical nuclei are amongst the sporoplasm. An iodinophilous vacuole is not revealed by staining with Lugol's solution.

Remarks

To date, four species of *Myxobolus* have been described in freshwater fishes from Australia. These are: *Myxobolus (Myxosoma) ogilbyi* Johnston and Bancroft, 1918 on the gill arches of *Macquaria ambigua;*

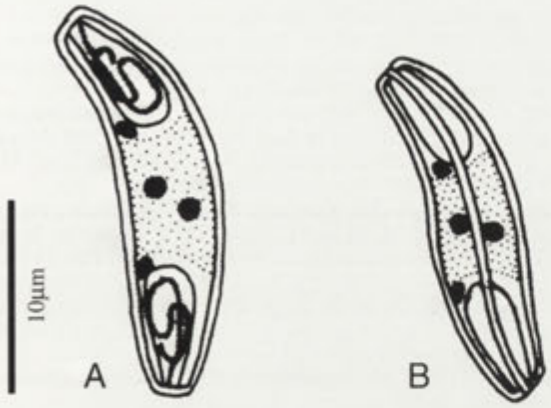


Fig. 3. Spores of *Sphaeromyxa nesogobii*. A - valvular view, B - sutural view

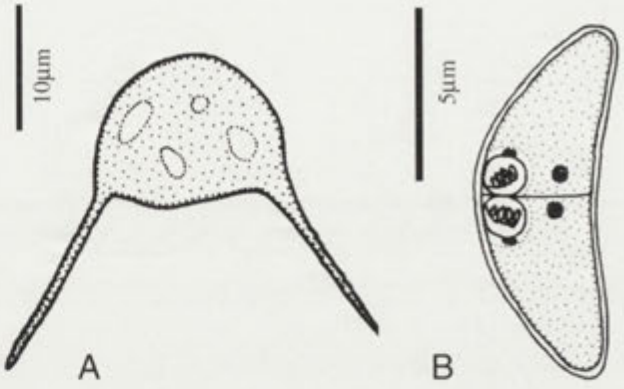


Fig. 6. *Ceratomyxa arripica* plasmodium (A), spore (B)

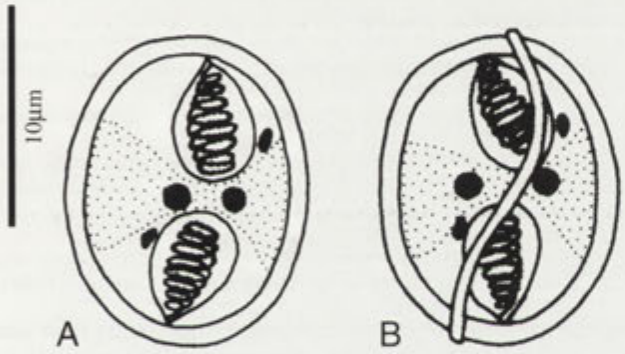


Fig. 4. Spores of *Zschokkella macrocapsula*. A - valvular view, B - sutural view

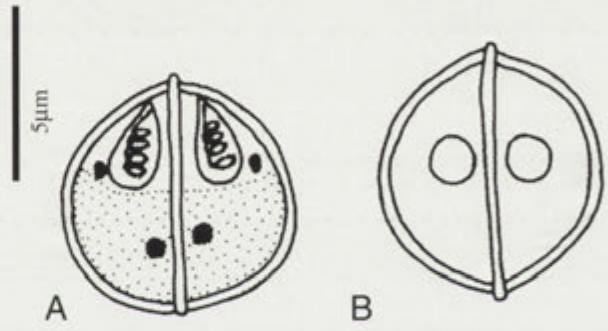


Fig. 7. Spores of *Sphaerospora aldrichettae*. A - valvular view, B - sutural view

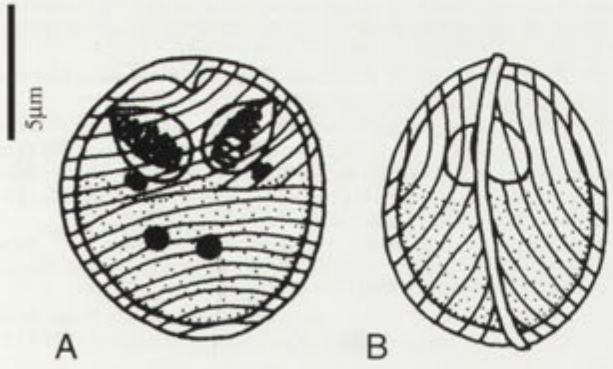


Fig. 5. Spores of *Ortholinea striateculus*. A - valvular view, B - sutural view

*M. plectroplites* Johnston and Bancroft, 1918 in the gall bladder and kidney of *Macquaria ambigua; M. gadopsii* Langdon, 1990 in the muscle and connective tissue of *Gadopsis bispinosus* and *G. marmoratus;* and *M. galaxii* Langdon, 1990 in the spinal cord of *Galaxias olidus.* Although the infection of Australian marine fish by unidentified *Myxobolus* species have been previously reported (Moser et al. 1989, Rothwell and Langdon 1990), the present species is the first formal description of a species of *Myxobolus* from Australian marine fish. This species can be readily distinguished from *M. plectroplites* in having no iodinophilous vacuole and from *M. ogilbyi* and *M. gadopsii* in the shape and dimensions of spores. Although the general appearance of *Myxobolus aldrichetti* is similar to that of *M. galaxii* and the dimensional ranges fall within those of that species, the present species differs from *M. galaxii* in its location and having only 4-5 triangular folds on the posterior margin of spore valves; while *M. galaxii* with 8-9 folds around. In a comparison of new species with other previously reported species from the world, the present species shows close resemblance to *Myxobolus cartilaginis* Hoffman et al., 1965 and *M. dermitis* Haldar et al., 1981 in the shape of spores. However, *M. cartilaginis* differs from *Myxobolus aldrichetti* in the larger dimensions of spores and polar capsules, and having 6-9 sutural markings at the posterior margin of the spore.

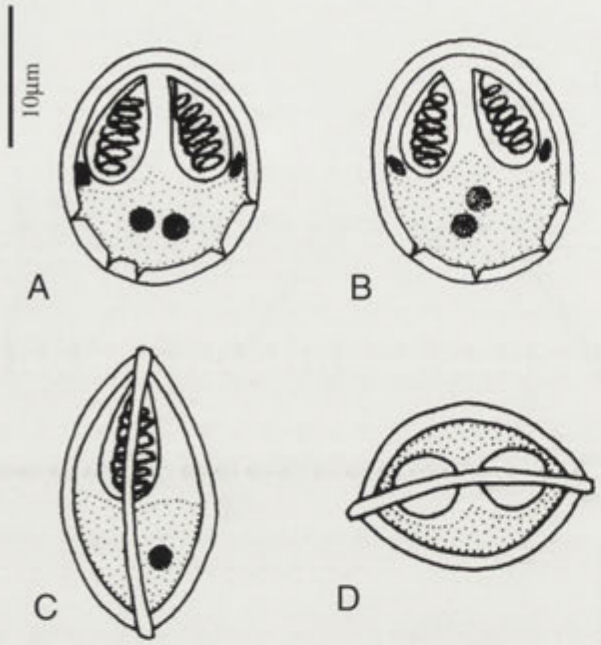


Fig. 8. Spores of *Myxobolus aldrichetti*. A-B - valvular views, C - sutural view, D - apical view

*Myxobolus dermitis* is distinguished from the species described here in completely lacking the folds of spore valves. Furthermore, *M. cartilaginis* and *M. dermitis* have not been found on the gills of fish: *M. cartilaginis* infected the cartilage of head and *M. dermitis* infected scales. *Myxobolus branchalis* Markewitsch, 1932 (Schulman 1966), reported from the gills of *Barbus barbus, B. barbus borysthenicus, B. brachicephalus caspius, B. capito conocephalus, B. capito* and *Mugil cephalus* is also similar in the shape to the present species. But it differs from *M. aldrichetti* in having smaller spores and polar capsules, and the presence of a triangular intercapsular process. Also there is no fold on the posterior margin of spore valves in *M. branchalis*. In view of these differences, the present form is described as a new species and the name *Myxobolus aldrichetti* is proposed following the host fish.

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**Book Review**

## Traité de Zoologie - Anatomie, Systematique, Biologie. Tome II, Fascicule 2 - Infusoires Ciliés edited by Pierre de Puytorac.

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In 1949 the first volumes (Tome VI and IX) of the "Traité", the still unsurpassed and most comprehensive post-war encyclopaedia on animals, appeared. Their founders under the leadership of Pierre - P. Grassé have hardly expected that it would take 45 years to (almost) complete this monumental work. Protozoologists in particular, who got "their" first volumes in 1952 (Tome I, Fascicule I: Phylogénie - Protozoaires: Généralites - Flagellés) and 1953 (Tome I, Fascicule II: Protozoaires: Rhizopodes, Actinopodes, Sporozoaires, Cnidosporidies) doubted that they ever would see the lacking volumes on the ciliates. New hope arose when **Tome II, Fascicule 1, Ciliés** appeared in 1984, containing an assembly of articles of varying up-to-datedness on cytology, reproduction, morphogenesis, biochemistry and ecology of ciliates, to mention only a few important topics. It took another 10 years for **Tome II, Fascicule 2, Ciliés**, now completing the protists with the treatment of the ciliate systematics. Congratulations to Monsieur Pierre de Puytorac, who probably did himself a great favour in finishing this project shortly before his retirement and also to the many contributors (mentioned below), who kept their shoulders to the wheel for years.

I think that such a comprehensive work on the systematics of ciliates can only be reviewed by describing its internal structure. The book starts with a general introduction to the systematics of the Phylum Ciliophora by Pierre de Puytorac (who also gives most of the short diagnosis (usually half a page) of the higher taxa listed below and marked with a star *. In his introduction, he discusses the benefits of comparative morphology and morphogenesis with particular reference to the structural organization of ciliate cortex. In conjunction with the ribosomal RNA data, he comments on the recent subdivision of the Ciliophora in three subphyla (Tubulicorticata, Filicorticata, Epiplasmatashowing a cortical microtubular lattice, an ecto-endoplasmic microfibrillar boundary, or a submembranous epiplasmic layer, respectively) - a concept first published by de Puytorac et al. in Comp. Rend. Acad. Sci. **316**, 716-720,(1993)

The following list shows the substantial core of the book together with the specialists who wrote the actual chapters on the various classes and subclasses. Their work will probably survive the never ending turmoil of macro-systematics.

Phylum **Ciliophora**
Subphylum **Tubulicorticata** *
Superclass **Postciliodesmatophora** *
Class **Karyorelictea** by Pierre de Puytorac
Class **Heterotrichea** by Michel Tuffrau and Pierre de Puytorac
Superclass **Spirotricha**
Class **Hypotrichea** by Michel Tuffrau and Anne Fleury

Class **Oligotrichea**
Order **Oligotrichida** and **Choreotrichida** by Michèle Laval-Peuto, Jean Grain and Gilbert Deroux
Order **Tintinnida** by Michèle Laval-Peuto
Superclass **Transversala** *
Class **Colpodea** by Wilhelm Foissner
Class **Plagiopylea** by Pierre de Puytorac
Subphylum **Filicorticata** *
Class **Litostomatea** by Jean Grain
Class **Vestibuliferea** by Jean Grain
Subphylum **Epiplasmata** *
Superclass **Ciliostomatophora** *
Class **Phyllopharyngea**
Subclass **Cyrtophoria** by Gilbert Deroux
Subclass **Chonotrichia** by Adrien Batisse
Subclass **Rhynchodia** by Pierre de Puytorac

Subclass **Suctoria** by Adrien Batisse
Superclass **Membranellophora** *
Class **Nassophorea**
Subclass **Prostomatia** by Pierre de Puytorac
Subclass **Nassulia** by Gilbert Deroux
Class **Oligohymenophorea** *
Subclass **Peniculia** by Pierre Didier and Pierre de Puytorac
Subclass **Scuticociliatia** *
Order **Philasterida** by Pierre de Puytorac
Order **Pleuronematida** by Pierre de Puytorac
Subclass **Peritrichia** by Jiri Lom and Pierre de Puytorac
Subclass **Hysterocinetia** by Pierre de Puytorac
Subclass **Astomatia** by Pierre de Puytorac
Subclass **Hymenostomatia** *
Order **Tetrahymenida** by Denis H. Lynn
Order **Ophryoglenida** by Pierre de Puytorac
Subclass **Apostomatia** by Pierre de Puytorac

(Experts will notice the elevation in rank of hysterocinetine ciliates (mostly endocommensals of oligochaetes), which were formerly placed among the thigmotrichous scuticociliates).

All major chapters are organized in the same way treating morphology, ultrastructure, morphogenesis, physiology and ecology first. The major systematic part follows and extends at least down to the genera. The book is copiously illustrated with 283 figures or plates, mostly composed of clear linedrawings arranged in subfigures. This is not the place for a lengthy discussion of the ranking of the various classes and subclasses (and the endings used). We all know what is meant.

I almost forgot to mention a short chapter by Mme Bonhomme-Florentin on the metabolism of rumen ciliates and their relation to rumen bacteria, a subject that would have been better placed in fascicule 1 of tome II.

One tremendous value of this book is that it gives a concise summary of the numerous papers which appeared in PROTISTOLOGICA (a journal not found at every university), the literally hundreds of notes published (and buried) in the Comptes Rendus de la Académie des Sciences, and dozens of "Thèses d'Etat" of many French ciliatologists.

Are there major shortcomings of this book? Not that I can think of, except that I would have liked to see a broader discussion of the possible phylogenetic interrelationship among the various classes and subclasses, which might have been a very slippery enterprise. One the other hand the chief editor felt very strongly about the propagation of the three subphyla (Tubulicorticata, Filicorticata, Epiplasmata), which I cannot accept for the moment. I think the recent approach by de Puytorac, Grain and Legendre using parsimony methods to reconstruct the phylogenetic tree of ciliates (Europ. J. Protistol. **30**, 1-17, 1994) shows the whole dilemma of ambiguity in this business. We are hardly better off with the molecular data of both 16 S rRNA and 25 S rRNA data as we begin to learn that even these molecular techniques (taken alone) will not solve the problem of early multifurcation at high taxa level.

So, what to do? The discussion will continue, but on what terms? Current founding policy greatly discourages teachers and scholars likewise to put great interest in the biology of organisms. We need to stop this attitude. Ciliates form the most marvellous example of diversity among protists. Take this book from **your** shelf and show it to your students, to encourage them to broaden their horizons!

**Christian F. Bardele, Tübingen, Germany**

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# Author Index: Acta Protozoologica 33 (1-4) 1994

# Subject Index*: Acta Protozoologica 33 (1-4) 1994

*See **Index of Taxonomic Names** of J. O. Corliss: An Interim Utilitarian ("User-friendly") Hierarchical Classification and Characterization of the Protists. *Acta Protozool.* **33**, 1-51

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