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Photophobic Responses in *Euglenina*. 1. Effects of Excitation Wavelength and External Medium on the Step-up Response of Light- and Dark-grown *Euglena gracilis*

Ewa MIKOŁAJCZYK

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

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Synopsis. This investigation establishes the light intensity thresholds for the step-up photophobic response in green and dark-grown cells of *Euglena gracilis* and compares them with the thresholds of buffer-rinsed cells.

At wavelength of 350-480 nm, the light intensity threshold is considerably lower in dark-grown cells than in green ones, above 480 nm their differences in sensitivity to an increase in light intensity diminish gradually. However, the action spectrum of photophobic responses is slightly broader in dark-grown cells.

When green and dark-grown cells are rinsed with a Mg-Ca-K buffer solution, their light intensity thresholds for the step-up response are lowered, in comparison to the thresholds of cells grown in culture medium. These decreased thresholds are associated with a broadening of the action spectrum for the step-up photophobic response (i.e., 350-550 nm). The greatest threshold differences between control and buffered cells occur between 350-480 nm, at longer wavelength, e.g., 525 and 535 nm, the differences in their response thresholds diminish. In dark-grown cells, the threshold may be decreased by a factor from 1.4 to 10, depending upon the excitation wavelength, and after some time may rise well beyond the pre-rinsing level by a factor from 1.5 to 33. In contrast, green cells may experience a transitory drop in the step-up threshold, which subsequently returns to its initial level.

The significance of these data in relation to problems of photoreception is discussed.

The green flagellate, *Euglena gracilis*, is known to react to sudden changes in incident light intensity (for terminology on these phobic responses see Diehn et al. 1977).

Cells with a stigma can respond to both decreases and increases in incident light intensity, while cells without a stigma are able to respond only to increases in light intensity. Thus, light-grown cells, containing a deeply colored stigma and chloroplasts, have both photophobic responses and exhibit both positive and negative phototaxis. Dark-grown cells, in which the transmittance of the stigma is about 70% (Benedetti et al. 1976), show both photophobic responses and about 50% of the level of photoaccumulation exhibited by green, wild-type cells, whereas permanently streptomycin-bleached cells with the stigma deprived of carotenoids exhibit only a step-up response and a negative phototaxis at any light intensity thus far tested (Ceccucci et al. 1976 b, Colombetti et al. 1981).

In the case of the step-up response, the stigma (when cells have one) determines the light intensity threshold. An increase in stigma transmittance, as occurs in dark-grown cells, causes a considerable decrease in the threshold for the step-up response, as compared with green cells. The threshold for this reaction falls to the level of that for the step-down response (Colombetti et al. 1981).

Lately, attention has been devoted to the lability of the light intensity threshold for the step-up photophobic response. In general, the threshold for the step-up reaction decreases when, for some reason, the step-down reaction is restricted or does not occur; this has been found in: (a) light-grown cells in lag phase of growth (Colombetti et al. 1981), (b) light-grown cells transferred to buffer solution (Mikołajczyk and Pado 1981), (c) cells in which the step-down reaction was inhibited with the detergent CTAB (Mikołajczyk and Diehn 1978).

Since a change of medium elicits a decrease in the step-up threshold in green cells (see above), it is of interest whether the same will happen in dark-grown cells, which have a lower threshold. This paper investigates (1) whether there are differences in the light intensity thresholds for the step-up photophobic response between green and dark-grown *Euglena gracilis* as a function of excitation wavelength, and (2) whether rinsing such cells with a buffer solution will evoke an even lower threshold for the step-up response, especially in dark-grown cells.

Materials and Methods

Green and dark-grown *Euglena gracilis* strain Z were grown in glutamate-malate medium (Greenblatt and Schiff 1959) at pH 3.5 and 24–25°C. Green cells were kept under continuous fluorescent cool-white light (1.0 W/m²). For experiments to determine the step-up thresholds in light- and dark-grown cells, five-day-old cultures were rinsed twice and resuspended in culture medium supernatant. For

similar experiments on the effects of rinsing with buffer, five-day-old cultures were gently centrifuged, washed twice in a solution containing 0.5 mM CaCl_2 , 0.125 mM MgCl_2 , 1 mM KCl and buffered to pH 7.1 with 2.5 mM Hepes-Pipes buffers and NaOH (see Doughty and Diehn 1979, 1982) and resuspended in the rinsing solution. Control cells were rinsed twice with culture medium supernatant. Prior to experimentation, the green cells were kept under dim red light and the dark-grown ones in darkness. The cell density did not exceed 10^4 cells/ml.

To establish the light intensity threshold for the step-up response at a given wavelength, at least 20 individuals were observed at each different light intensity. The differences in sensitivity between light and dark-grown cells and between control and rinsed cells to increased light intensity were determined by testing at least 100 individuals of each group at each wavelength. The cells were stimulated only once with monochromatic light and then discarded. Tumbling in 100% of the examined cells was taken as "standard" response. The minimum light intensity necessary to elicit the standard photophobic response was used to measure cell sensitivity and to establish the threshold of this response.

The photophobic responses were observed in a small glass chamber 15 mm in diameter and 1.5 mm deep under a NU microscope (Carl Zeiss Jena). Background illumination at 690 nm, which is not perceived by the cell, was applied through the objective lens (Fig. 1). An HBO 101 W halogen lamp mounted to

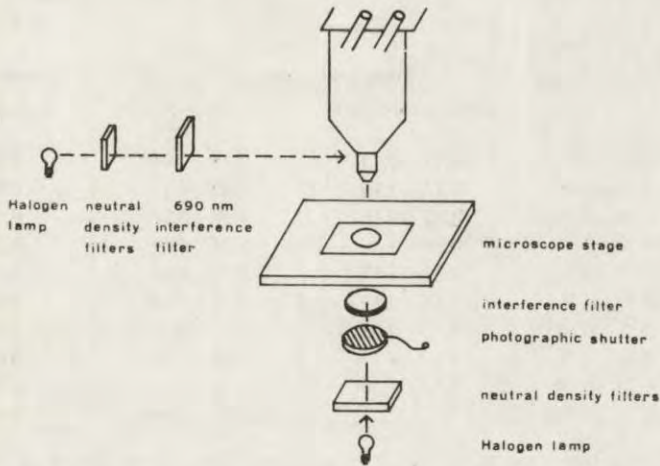


Fig. 1. Schematic diagram of optical system used for investigating step-up photophobic response as a function of excitation wavelength

the microscope was used as the light source to indicate the photophobic response. Light intensity was controlled by inserting neutral density filters and measured by an EG and G Radiometer, Type 550.

Light stimuli were applied through a condenser controlled by a photographic shutter. Monochromatic light (half-width 4–11 nm), of variable wavelength and intensity, was obtained by inserting interference filters (Carl Zeiss Jena) between the photographic shutter and the microscope stage.

Results

Step-up Response Thresholds in Light- and Dark-grown Cells

Observations of the light intensity threshold for the step-up photophobic response in green and dark-grown cells were carried out one hour after rinsing cells with culture medium. At wavelengths of 350–480 nm, the threshold was considerably lower (by a factor from 3 to 5) in dark-grown cells than in green ones. Above 480 nm, their differences in sensitivity to an increase in light intensity diminished, with a sharp drop in sensitivity commencing at 510 nm, where the threshold difference between green and white cells was by a factor of 2, and at 525 nm by a factor of 1.4 (Table 1, Fig. 2).

Table 1

Changes in light intensity thresholds for the step-up response in light- and dark-grown *Euglena* as a function of excitation wavelength and external medium

Wave-length nm	R.T. = $\frac{I_{\text{white}}}{I_{\text{green}}}$	R.T. = $\frac{I_{\text{buffered}}}{I_{\text{control}}}$		R.T. = $\frac{I_{\text{control}}}{I_{\text{buffered}}}$
		Threshold decrease		Threshold increase
		Light-grown cells	Dark-grown cells	Dark-grown cells
350	0.25±0.27	0.13±0.13	0.16±0.06	0.08±0.04
375	0.26±0.18	0.15±0.11	0.49±0.17	0.06±0.11
400	0.28±0.15	0.12±0.10	0.40±0.20	0.03±0.01
425	0.29±0.31	0.36±0.20	0.21±0.06	0.03±0.01
450	0.22±0.28	0.31±0.17	0.10±0.06	0.07±0.07
475	0.27±0.26	0.34±0.27	0.22±0.10	0.04±0.07
500	0.33±0.14	0.58±0.03	0.47±0.01	0.05±0.02
510	0.48±0.23	0.59±0.17	0.54±0.09	0.06±0.02
525	0.70±0.09	0.72±0.20	0.70±0.09	0.16±0.14
535	+	++	0.69±0.00	0.64—

R.T — Relative threshold is the ratio of the light intensity threshold of the dark-grown cells (I_{white}) to that of light-grown (I_{green}), (column 1), or of the buffered cells (I_{buffered}) to the control cells (I_{control}) (columns 2, 3), or of the control cells to the buffered cells (column 4).

+ At this wavelength, only dark-grown cells showed a step-up response.

++ Control cells did not respond at this wavelength, so a ratio could not be determined.

At a wavelength of 525 nm, not all light-grown cells reacted to stimulation with light of a maximal intensity of $5.0 \times 10 \mu\text{W}/\text{cm}^2$. For example, of the seven experiments carried out on a total of 140 cells, 60 individuals did not show the standard step-up response. All dark-

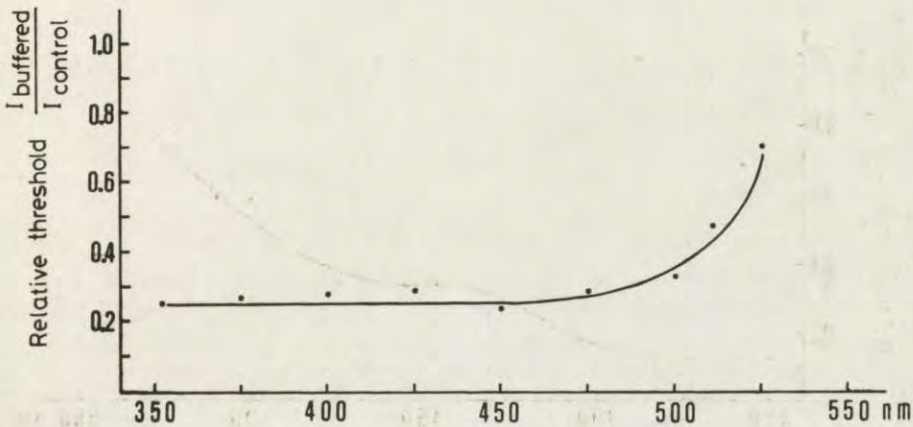


Fig. 2. Modification of the light intensity threshold for the step-up photophobic response in dark-grown *Euglena* as a function of excitation wavelength. Relative threshold is the ratio of the light intensity threshold for the step-up response of dark grown cells (I_{white}) to that in light-grown cells (I_{green})

grown cells, however, respond with a step-up reaction to a markedly lower light intensity.

At a wavelength of 535 nm and a maximal light intensity of $4 \times 10 \mu\text{W}/\text{cm}^2$, the standard step-up reaction was not observed in any of seven experiments with green cells, but in dark-grown cells it was observed in four of the seven experiments performed, (i.e., in 80 of 140 cells).

At a wavelength of 550 nm, cells of neither type reacted to $9 \times 10 \mu\text{W}/\text{cm}^2$, the maximal intensity applied.

Modification of Light Intensity Thresholds for the Step-up Response in Buffered Light-grown Cells

When green cells were rinsed with a Mg-Ca-K buffer solution, their step-up response thresholds were lowered, in comparison to those of control cells rinsed with culture medium. After such transitory decreases in the threshold, it subsequently returned to its initial level but never exceeded it. The greatest threshold differences (by a factor of 9) were found in the spectral range of 350–400 nm (Table 1, Fig. 3). Slightly less sensitivity to light stimulation was noted between 425 and 475 nm. Within this range, the light intensity threshold of buffer-washed cells differed from the controls by a factor of 3. At longer wavelengths the sensitivity of the buffered cells to light stimulation decreased. For example, at 500–525 nm light intensity threshold of the buffered individuals was lower only by a factor of 1.4.

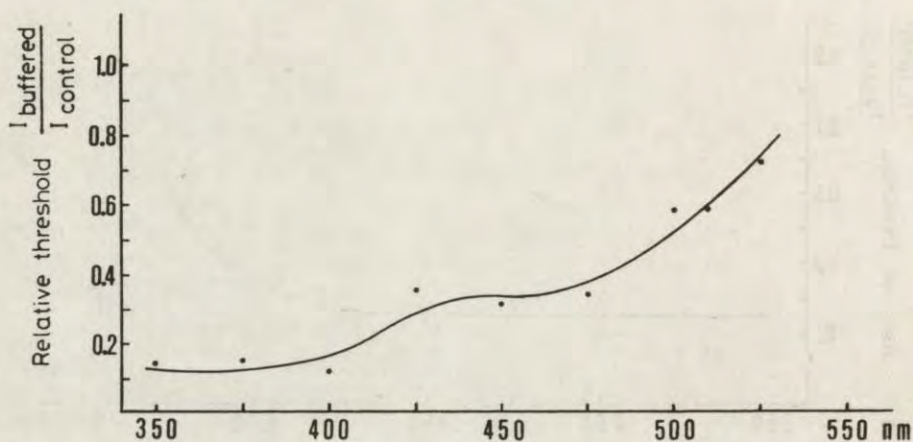


Fig. 3. Decrease of light intensity threshold for the step-up response in light-grown buffer-rinsed *Euglena* as a function of excitation wavelength. Relative threshold is the ratio of the light intensity threshold of the buffered cells (I_{buffered}) to that of the control cells (I_{control})

At a wavelength of 525 nm and a maximal stimulation of $5 \times 10 \mu\text{W}/\text{cm}^2$, the standard step-up reaction was not observed in three of seven experiments with 160 control cells. In contrast, all 160 buffered cells examined showed a standard step-up response, with a light intensity threshold lower than in the controls by a factor of 1.4.

At 535 nm and a maximal intensity of $4 \times 10 \mu\text{W}/\text{cm}^2$, a step-up response was not elicited in any of 8 experiments (i.e., at least 160 cells observed) carried out with controls, whereas the standard reaction was produced in ca. 50% of the buffered cells observed. At a wavelength of 550 nm, neither type of cell reacted to light of the maximal intensity applied ($9 \times 10 \mu\text{W}/\text{cm}^2$).

Modification of Light Intensity Thresholds for the Step-up Response in Buffered Dark-grown Cells

The exchange of culture medium for buffer with K^+ , Mg^{2+} and Ca^{2+} ions usually elicited a decrease in the light intensity threshold for the step-up response in dark-grown cells (Table 1, Fig. 4-bottom). This lowered threshold appeared 20–30 min after rinsing the cells and persisted for 1–4 h.

At wavelength of 350 nm the greatest decreases in the step-up response thresholds occurred (by a factor of 10). At 375 nm the threshold of the buffer-rinsed cells was decreased by a factor of 2. At wavelengths longer than 450 nm, the threshold in buffered cells was

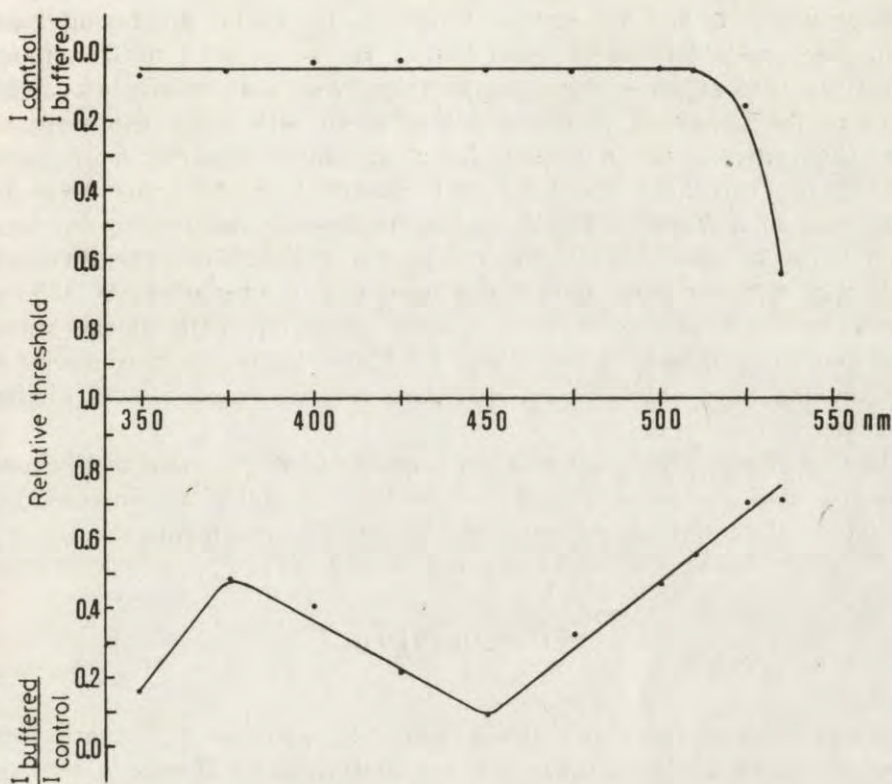


Fig. 4. Modification of light intensity thresholds for the step-up response in dark-grown buffer-rinsed *Euglena* as a function of excitation wavelength. Relative thresholds are the ratios of either the controls (I_{control}) to the buffered cells (I_{buffered}), (threshold increase, top curve) or the reverse (threshold decrease, bottom curve)

progressively higher, and at 525 nm differed from the control by a factor of 1.4. In all experiments carried out at 525 nm a standard step-up response was observed in both the buffered cells and the controls.

At 535 nm and a maximal intensity of $4 \times 10 \mu\text{W}/\text{cm}^2$, a step-up response was elicited in one third of the control and test organisms (i.e., 40 of 120 cells of each type), with the threshold in buffered cells lowered by a factor of 1.4. Interestingly, a step-up response occasionally occurred in buffered cells at 550 nm and a maximal intensity of $9 \times 10 \mu\text{W}/\text{cm}^2$, but it was never observed in the controls at this wavelength and intensity. This broader action spectrum for step-up responses appeared in cells in which the step-up threshold was exceptionally low at other wavelengths.

It is also note worthy, that the step-up threshold in all rinsed

dark-grown cells did not simply return to its initial prerinsing level, as in green cells, but rose beyond it by a factor from 1.5 to 33, a result indicating that at some wavelengths there was a difference of ca. 300 times in the threshold levels, and that these cells were less sensitive than the controls to increased light intensity. Several hours after rinsing, the threshold stabilized and remained at the same level for more than 24 h (Table 1, Fig. 4-upper). At 350–510 nm the step-up threshold is ca. 30 times higher than the controls; at 525 nm, the threshold level was differed from the control only by a factor of 6. At 535 nm a step-up response was elicited in only about one fifth of the rinsed cells and their threshold level was 1.5 times higher than controls. At 550 nm and maximal light intensity, no step-up response was elicited in rinsed or control cells.

In two of the seven experiments carried out with rinsed dark-grown cells the step-up threshold did not decrease initially but instead, immediately after rinsing, rose sharply beyond the prerinsing level.

Discussion

Stimulation of green and dark-grown cells with monochromatic light of wavelengths of 350–550 nm showed that widest differences between green and dark-grown cells in the light intensity threshold for the step-up response occurred from 350 to 480 nm, but as in the case of the raised threshold in dark-grown cells, could be extended to 510 nm. At longer wavelengths, the threshold differences diminished.

The decrease in the threshold in buffered-rinsed cells is associated with a broadening of the action spectrum for the step-up response. It was noted that in some conditions the step-up response can be observed even at wavelength 550 nm. This result indicates that elicitation of a cell reaction to an increase of light intensity is dependent on the intensity of stimulating light and not on the wavelength within the range 350–550 nm.

There is considerable evidence that flavins are involved in the photoreactions of *Euglena* (Barghigiani et al. 1979, Checcucci et al. 1976 a, b, Colombetti et al. 1982, Diehn 1969, 1979, Doughty and Diehn 1980). If we assume that flavins are, in fact the photoreceptor pigments for the step-up response, it is difficult to explain the sensitivity of cells in the spectral range beyond 520 nm, since it is known that flavins or flavoproteins do not absorb beyond this wavelength. Thus, there may be in *Euglena* an accessory photopigment for the step-up response, as is the case, for instance, in *Halo-*

bacterium halobium (Hildebrand 1973). According to the observations of Colombetti and Lenci (Colombetti et al. 1982) the step-up reaction may be evoked only by PFB stimulation; such an accessory pigment would have to be close to the PFB, and located perhaps in the flagellar membrane.

Observations to date on the decreased step-up response threshold indicate that it may be either permanent or transient. A permanent decrease, for example, may be observed in dark-grown cells and is associated with partial decoloration of the stigma, giving it enhanced transmittance. The step-up response threshold is decreased as long as cells are maintained in darkness, thus preventing renewed carotenoid synthesis in the stigma. A transient decrease of the step-up threshold is elicited by changes of the external medium. For example, changes in the chemical composition of the medium, may cause a transient disappearance of the step-down response (Doughty and Diehn 1979, 1983, Mikołajczyk and Pado 1981) as well as decrease of the step-up response threshold (Mikołajczyk and Pado 1981).

Investigation of photophobic reaction of light-grown *Euglena gracilis* indicate that the decrease of the threshold for the step-up response is associated with a partial reduction or complete inhibition of the step-down response (Mikołajczyk and Diehn 1978, Colombetti et al. 1981, Mikołajczyk and Pado 1981). As soon as the cells recover the ability to perform the step-down response, the threshold for the step-up reaction increases. According to Colombetti et al. (1981), these data suggest the existence in *Euglena* photosystem of two phototransduction chains — a separate one for the step-up and step-down response. The effect of rinsing on the expression of the step-up response in dark-grown cells seems to be much more complicated. The same cells may show a decrease, beyond the prerinsing level, followed by an increase of the light intensity threshold. The difference in the step-up response thresholds between light- and dark-grown cells seems to be closely related to the chemical composition of the external medium, however, the mechanism of this phenomenon remains to be elucidated.

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Studies on Cell Adaptation: Effects of a Cycloheximide
on *Euplotes minuta*

M a u r y l a K I E R S N O W S K A

Institute of Zoology, Warsaw University, P-00-927/1, Warsaw, Poland

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Synopsis. The average daily fission rate of *Euplotes minuta* (homozygous strain A-23) was reduced in relation to the concentration of cycloheximide (CHX 0.1-5 µg/ml) administered either for 4 days to newly replicated cells or every other day for 29 days to the same cell culture. In a given CHX concentration the fission rate of cells was always reduced to the same value when compared to appropriate control cells. Cultures in the presence of CHX entered the stationary phase at a lower cell density than control cultures. The observed slow-down or/and full inhibition of *Euplotes minuta* developmental processes were due solely to the drug concentration and not the time of drug treatment. The effects induced by cycloheximide were fully reversible after the cells had been transferred to a control medium without CHX. The inhibitor did not bring about any change in the average macronuclear DNA content and a considerable variability in the DNA content of single cells is retained. In spite of this variability, prolonged (up to 29 days) presence of the CHX does not result in any specific modification in the drug resistance of the total cell population. The possibility of a positive selection of a resistant macronuclear variant of homozygous *Euplotes* in the presence of low doses of CHX, are discussed.

It was demonstrated that *E. minuta*, a hypotrich ciliate, is not capable of endogenous recovery from the effects of actinomycin D (Kiersnowska and Jasińska 1984, accompanied paper). In order to check whether the response of *Euplotes* is independent of the kind of inhibitor, i.e., its chemical structure and mode of action, the reaction of this ciliate to cycloheximide (CHX), an inhibitor of protein biosynthesis (Vasquez 1979), has been tested.

It has been observed that ciliates such as *Chilodonella* (Kiersnowska 1982) or *Tetrahymena* (Frankel 1979, Hayer and Frankel 1971), capable of recovery from the effects of many drugs,

react to CHX presence with an initial inhibition of fission and then reinitiation of the process at a normal level. In treated cells the first cell division is delayed for a period which corresponds to that of drug-induced fission inhibition, after which all cells simultaneously become insensitive to the inhibitor, although it is present in the medium. Generally, it is characteristic for this reaction, inhibition of culture growth lasts no longer than the time for one to two cell generations. In *Euplotes*, results from the present investigation demonstrate that such rapid return to pretreatment generation times in the presence of inhibitor does not occur. In four-day experiments CHX brings about only diminution of culture growth, despite that the duration of the experiment corresponds to seven control cell generation times. In view of these results, it was necessary to examine whether a gradual increase of the average fission rate occurs during prolonged drug treatment. The phenomenon could occur if one or several cells were more resistant than the other and would initiate a line of equally resistant cells. It is possible to isolate such a cell in the laboratory, without an action of mutagen. Schimke et al. (1978, 1980) demonstrated that among mammalian cells in tissue culture 1 cell per 100 000 is resistant to a certain dose of methotrexate. The resistance of the cell and its progeny is due to a chance, selective amplification of a gene whose product is attacked by the inhibitor. A similar phenomenon is observed in bacteria, with a frequency as high as 1/1000 (Anderson et al. 1976).

The macronucleus of ciliates contains many copies of most genes. This natural amplification is particularly high in hypotrich ciliates, in which the macronuclear DNA is arranged in gene-sized pieces (Lawn et al. 1978; Lipps and Steinbruck 1978). It is estimated that each DNA sequence appears in about 1000 copies (Lauth et al. 1976) and r-DNA genes are 100 times more highly repeated than the bulk of macronuclear DNA sequences (Swanton et al. 1980). An amitotic fission of this macronucleus probably results in an irregular distribution of gene-copies among progeny nuclei, but the great number of copies present is a sufficient guarantee against aneuploidy.

As a result of the irregular distribution of genetic material to sister cells, the difference of macronuclear DNA content in *Euplotes* can be as high as 72%, and the average difference for sister cells is 11.6% (Witt 1977). It is not unlikely that some cells will contain many more copies of a gene. Thus it is equally possible that there will appear by chance a cell containing a sufficiently large number of genes whose product(s) would decrease or totally neutralize the effect of non-lethal concentrations of CHX. Such macronuclear variant would divide faster

than the sensitive cells. However, the basic question is whether, under conditions of permanent exposure to CHX, the progeny of this cell will retain the high number of these genes which neutralize the drug effect. If so, then the resistant cells should outnumber other cells in the culture, and the average daily fission rate should increase in time with the presence of CHX in the culture medium.

The present study deals with the investigation of the fission rate of *Euplotes minuta* cells, strain A-23, growing in the continuous presence of CHX.

The cells of this strain underwent cyclical autogamy every fourth week which proves that the observed cells were fully homozygous. During autogamy, either duplication or deletion of genetic material may occur but the probability that a spontaneous genetic mutation would appear, was practically nil.

Materials and Methods

The marine ciliate, *Euplotes minuta*, strain A-23 was cultivated in synthetic sea water and fed on *Dunaliella* sp. The manner of incubation has been described previously (Kiersnowska and Jasińska 1984). The untreated cells (controls) and cycloheximide treated cells always received the same amount of *Dunaliella*. The *Dunaliella* culture made up a third of the total volume of the medium. The appropriate concentrations of CHX (Koch-Light Lab. LTD) were prepared just prior to the experiment. Preliminary studies demonstrated that CHX at concentrations lower than 7 µg per ml were not lethal to cells. The effect of non-lethal doses of CHX on the fission rate of *E. minuta* was analyzed on the basis of the daily fission rate of the cells (Kiersnowska and Jasińska 1984). Single cells isolated at random from the mass culture were transferred individually (clones), or in groups of 10, to depression slides, where the final volume of the medium was 0.6 or 1 ml, respectively. Two types of experiments were carried out:

(1) four-day experiments in which cells from control cultures 12–36 h old were transferred to media with or without CHX (0.1, 0.5, 1, 2 or 3 µg per ml) and growth of clones was tested every 24 h up to the fourth day;

(2) long-term experiments in which a control culture 36 h old was employed to initiate other mass cultures then cultivated for 7, 17 or 29 days in media with or without CHX (0.1, 1, 2, 5 g per ml). The initial number of cells in each control culture was between 1500 and 2000 (in 3 ml). Every other day the same number of cells was transferred to an analogous fresh medium after having been rinsed in sea water with or without CHX. After different exposure times to the inhibitor, clones or groups of ten-cell were replicated and their number controlled on subsequent days or after 48 h of growth.

Means and standard deviation of fission rates for a single series of measurements or the dispersion of measurements about a weighted average mean for several series together have been expressed (Armitage 1978).

The Control of the Process of Autogamy

The cells of *E. minuta* are mature for autogamy after 50–75 divisions (Siegel and Heckmann 1966). The occurrence of this sexual process was confirmed on the basis of observation of living cells and by staining DNA using the method of Dippell (1955). The cells that have undergone autogamy are smaller, slightly oblong and have a characteristic light spot in the middle.

Measurements of the Macronuclear DNA Content

Cells in division were isolated and 30 min later fixed with methyl-alcohol and formaldehyde, 9:1 respectively. The Feulgen method was employed for DNA with the use of pararosaniline (Elftman 1959). The hydrolysis time of 12 min was determined on the basis of photometric measurements of cells hydrolyzed for 5–20 min prior to staining. These measurements were made with a ZETOPAN microscope equipped with a Reichert microphotometric attachment, with a wavelength of 560 nm. The content of DNA in arbitrary units (AU) was calculated by the formula:

$$AU_{MA} = E_{MA} - E_C/S_{MA},$$

where: E_{MA} — the extinction mean of six independent measurements of the macronucleus, E_C — the extinction of cytoplasm, S_{MA} — the macronucleus area measured in arbitrary units: the shape of every macronucleus was sketched and then its area measured with a planimeter.

Results

Preliminary Experiments

Investigation of the degree of synchrony of cell fission (Table 1) and individual generation times (Table 2) demonstrated that CHX disturbs neither the asynchrony of divisions nor the natural variation of generation times. Concurrently with an increase of inhibitor concentration, one observes a reduction in the division frequency and a prolongation of generation time, including the cytokinesis period. To make sure that these effects were only the consequence of direct inhibition by CHX, it was necessary to rule out possible secondary effects such as modification of the inhibitor during the several days of the experiment, and detrimental effects of CHX on the flagellate food organism.

It was demonstrated that CHX activity was not significantly reduced for up to 3 days in cell cultures. Untreated ("naïve") cells were transferred into media to which CHX at a concentration of 1 $\mu\text{g}/\text{ml}$ had been added 1, 2 or 3 days previously and from which all ciliates undergoing divisions had been removed. These "naïve" cells divided at the same rate as other "naïve" cells introduced into fresh medium containing similar CHX concentrations.

Table 1

Frequency of cytokinesis stage in cultures of *Euplotes minuta* growing in the presence of cycloheximide

Medium	Periods (in hours)				
	0-12	12-24	24-36	48-60	60-72
Control	0.96±0.68	1.20±1.00	1.58±0.55	0.70±0.46	1.23±0.80
CHX 1 µg/ml	0.50±0.60	0.48±0.37	1.10±0.70	0.38±0.41	0.43±0.41
CHX 2 µg/ml	0.50±0.54	0.25±0.30	0.50±0.70	0.25±0.35	0.43±0.53
CHX 3 µg/ml	0.42±0.42	0.16±0.28	0.15±0.35	0.18±0.30	0.13±0.35

From the mass culture samples of 150-300 cells were taken every two hours, and cells in cytokinesis were counted. Any significant difference of dividing cells number between samples does not occur. Data represent a mean percentage (± SD) of dividing cells per sample in sequential 12 h periods.

Table 2

The average times of generation and cytokinesis of *Euplotes minuta* treated and untreated with cycloheximide

Medium	Generation time (h min ± SD)				Duration of cytokinesis (min s ± SD)	
	min.	max.	n	mean	n	mean
Control	7 ⁰⁵	11 ³⁶	33	9 ¹⁰ ±1 ¹⁰	20	8 ⁴⁰ ±1 ⁴⁸
1 µg/ml CHX	12 ²⁵	16 ⁴⁸	28	14 ²⁰ ±1	24	11 ¹⁵ ±2 ⁴⁵
2 µg/ml CHX	19 ³⁴	24	27	21 ³⁰ ±0 ⁵⁴		

Dividing cells were transferred from 48 h old culture (control and with 1 or 2 µg CHX/ml) to fresh medium (without or with CHX respectively), the time was measured from cytokinesis completion to the beginning of the next cytokinesis of sister cells. In the measurements of the duration of cytokinesis, cells in the V phase of fission morphogenesis (Wisse 1965) were selected.

The effect of CHX at 1-5 µg/ml on *E. minuta* food organism was assessed on the basis of:

(1) a comparison of the daily fission rate of ciliates previously starved for 24 h and then fed on *Dunaliella* untreated or treated with CHX at 2 or 5 µg/ml for 48 h prior to the experiment (Table 3),

(2) observation of the number of flagellates incapable of active movement in the presence of CHX (Table 4), since *Euplotes* does not feed on immobile flagellates (Heckmann pers.com.).

The daily fission rate of ciliates fed on CHX-treated and non-treated *Dunaliella* was the same, and the mobility of *Dunaliella* was markedly affected only after 2 days in the presence of the drug at concentration of 2 or 5 µg/ml. Approximately half of the flagellates do not swim actively. Therefore an additional experiment was carried out

Table 3

The effect of CHX pretreated food (*Dunaliella* sp.) on the daily fission rate starved *Euplotes minuta*

Number of experiment	Kind of food	Daily fission rate (\pm SD), f02 following days		Average daily fission rate (\pm SD) for days I and II
		I	II	
1	D	1.20 \pm 0.25	2.5 \pm 0.10	1.85 \pm 0.07
	D ₅ CHX	1.21 \pm 0.25	2.36 \pm 0.11	1.79 \pm 0.007
2	D	—	—	1.95 \pm 0.06
	D ₃ CHX	—	—	1.99 \pm 0.06
	D ₅ CHX	—	—	1.99 \pm 0.05

Dunaliella growing 48 h in the control medium (D) and in medium with 5 μ g/ml CHX (D₅CHX) or 3 μ g/ml CHX (D₃CHX) were rinsed with sea water and added to starved *Euplotes minuta* clones. The number of clones in each series was 10. In the experiment 2, cells were counted only after 48 h.

Table 4

Effect of cycloheximide on *Dunaliella* motility

Days \ Medium	1	2	3	4
	Control	—	—	—
CHX 1 μ g/ml	—	—	-/+	-/+
CHX 2 μ g/ml	—	-/+	++	+++
CHX 5 μ g/ml	—	-/+	++	++++

As criterium of CHX effect on *Dunaliella* motility the cessation of cell swimming (the settled of flagellates at the bottom of depression slide occur) was accepted. Symbols: "—" the lack of nonswimming cells; "-/+", "++" ... "++++" an increasing number of nonswimming cells agglomerate at the bottom.

designed to test the growth of clones in culture medium containing 1/2 the normal amount of *Dunaliella*. The fission rates after 1, 2 or 3 days of growth did not differ statistically from those in the medium containing the normal amount of food. However, due to the adverse effects of CHX on *Dunaliella*, it was necessary to renew the media every 2 days during long-term experiments.

Four-day Experiments

The course of the growth of the *Euplotes* clones in control medium and in medium with CHX (0.1–3 μ g/ml) is illustrated in Fig. 1. During 4 days of experiments the control cells divide 7 times and the phase

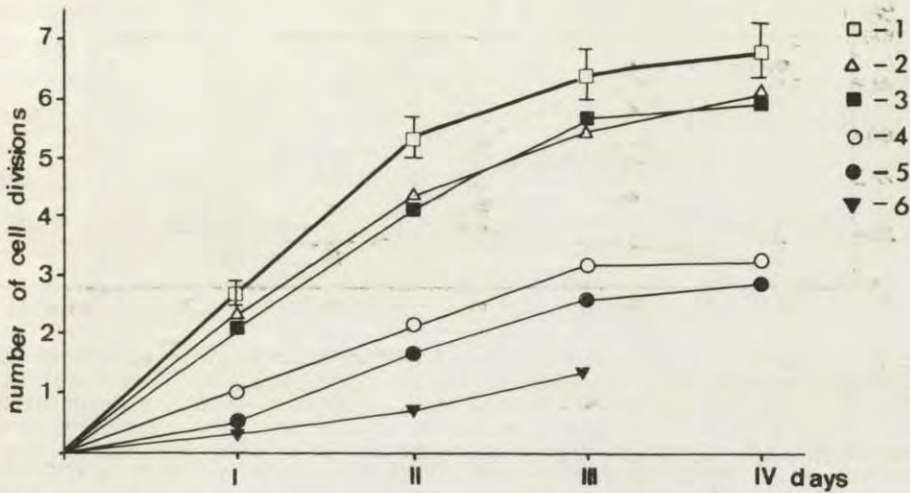


Fig. 1. Effect of cycloheximide on the growth of *Euplotes minuta* clones. The number of clones were: control (1) — 177, 0.1 µg/CHX/ml, (2) — 61, 0.5 µg CHX/ml (3) — 54, 1 µg/ml (4) — 34, 2 µg CHX/ml (5) — 42, 3 µg CHX/ml (6) — 53

of the most intensive growth (log phase) lasting two first days is followed by both the decelerating (third day) and next stationary phase (fourth day). The growth of the CHX treated clones is reduced in relation to the concentration of the drug. The CHX at the concentrations of 0.1 and 0.5 µg/ml only slightly affects the course of clonal growth. The fission rate of the cells slightly dropped and duration of the clonal growth phases are the same as in control conditions. The effects of the higher doses of the CHX are more evident. At the 1 or 2 µg CHX/ml the phase of the most intensive growth is one day longer than that of the control, and it is directly followed by the stationary phase. The course of the clonal growth in the presence of 3 µg CHX/ml is not analyzed here, since on the fourth day some effects of the CHX on the fed have been observed (e.g., the aggregation of the flagellates on the bottom of depression slides was visible).

It is very characteristic for the clonal growth in the CHX (0.1–2 µg/ml) that the stationary phase begins in nearly the same period as in control clones, in other words, independently of the cell number. The fact, that at the given concentration the daily fission rate of the CHX-treated cells subject to only slight changes until the stationary phase, strongly suggests the lack of the *Euplotes* capacity to endogenous recovery. Since the reaction of the recovery is generally manifested by the return to the pretreatment generation times, the comparison of the daily fission rate of the CHX-treated cells to the mean daily fission rate of the control cells during their log phase of the clonal growth, was

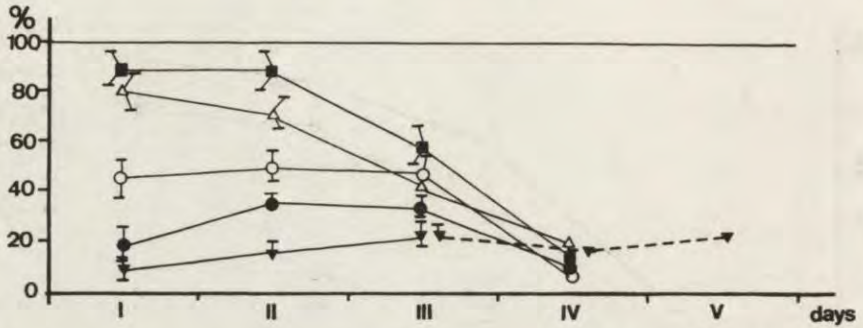


Fig. 2. Comparison of daily fission rate of CHX-treated *Euplotes minuta* to the fission rate logarithmically growing non-treated cells. Each point shows the ratio of the daily fission rate of CHX-treated cells on sequent days of experiment to average daily fission rate of respective control cells in their most intensive growth phase. Fission rate of control cells is expressed as 100%. Dotted line demonstrated the results of the experiment where single cells from medium with 3 μg CHX/ml were transferred after 2 days to fresh medium with the same dose of drug, other symbols as in Fig. 1

made. Cells treated with CHX (0.1–3 $\mu\text{g}/\text{ml}$) are never observed to overcome inhibition and divide at the rate characteristic of the control cells during two first days of the clonal growth (Fig. 2). A detailed analysis of the results of four days experiments provide additional important information:

(1) The fission rate of control cells depends on their age relative to their sexual cycle. Thus the average daily fission rate during the log-phase is highest (i.e., about 3 divisions per 24 h in the second or at beginning the third week following autogamy (data not presented).

(2) The average daily fission rate of both control cells and CHX treated cells are subject to analogous changes, i.e., as control cell fission rate increases, the rate in cells exposed to CHX increases too, and likewise decreased rates occur concomitantly in both groups of cells. However, the reduction of the log-phase cell fission rate at a given concentration of CHX is always of the same value in relation to the corresponding control (at 0.1 $\mu\text{g}/\text{ml}$ 15–20% lower than the control and 45–50% lower at 1 $\mu\text{g}/\text{ml}$). Since the growth of clones in the presence of CHX depends on the physiological conditions of the cells during their exposure to the drug, the action of some endogenous factors affecting cell divisions are not masked by the low doses of CHX employed.

(3) Although the average fission rate of CHX-treated clones was lower than in controls, some clones treated at 0.1 and 0.5 $\mu\text{g}/\text{ml}$ grew at the same rate as their corresponding controls. No such cases were reported among 129 clones cultivated in higher concentrations of the inhibitor. This indicates that the difference of the daily fission rate of

control cells and cells growing in 0.1 and 0.5 $\mu\text{g/ml}$ CHX is too small to examine the possibility of a positive selection of resistant lines. On the other hand, both the short duration of these experiments and the low number of cells tested at 1.2 or 3 μg CHX/ml made detection of any resistant cells difficult. In these reasons a series of long-term experiments, in continuous presence of low doses of CHX in culture medium, have begun.

Long-term Experiments

Three separate experiments were made on the 17th, 15th and 5th days after ciliates had completed autogamy. The experiments lasted respectively 7, 17 and 29 days. The results obtained demonstrate that the daily fission rate does not increase with increasing time and number of generations in a medium containing cycloheximide.

The most complete data were obtained in experiment No. 3 (Fig. 3). The daily fission rate of control cells and cells exposed to CHX at 1 and 2 $\mu\text{g/ml}$ were subject to parallel changes up to the 14th day. When the control cells underwent autogamy and post-autogamous transformations, the fission rate of CHX-treated cells decreased. From the 21 day at 1 μg CHX/ml and from the 14 day at 2 $\mu\text{g/ml}$ divisions per 24 h becomes more or less stable.

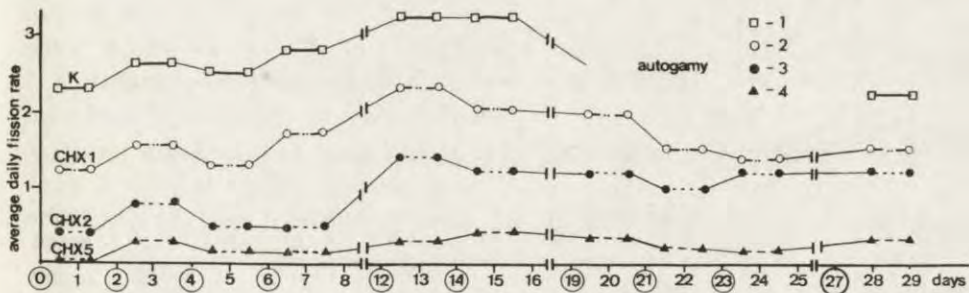


Fig. 3. Effect of long-term (29 days) presence of cycloheximide on average daily fission rate of *Euplotes minuta*. On 0, 2 ... and 27 day of experiment, 10 cells were transferred into depression slides (2 or 4 for each concentration), number of cells was noted after 48 h, each counted cells was eliminated. 1 — control cells, 2 — cells treated 1 μg CHX/ml, 3 — cells treated 2 μg CHX/ml, 4 — cells treated 5 μg CHX/ml

The effects of 5 $\mu\text{g/ml}$ are so strong that the changes in the daily fission rate are very small.

Two other experiments on CHX were carried out. In the beginning of both experiments, i.e., at "0" time of drug treatment, and then, re-

spectively, after 2, 3 or 4 days (experiment 1) or 1, 3, 8, 11 or 14 days (experiment 2), 15 clones were incubated in CHX at 0.1 or 1 $\mu\text{g}/\text{ml}$. The clone growth was tested every day up to the fourth day. In the most intensive phase of clone growth, the average daily fission rate at 0.1 μg CHX/ml was reduced by about 20% compared to the corresponding control cell fission rate. At 1 μg CHX/ml the fission rate was about 40% lower and during autogamy of control cells the decrease amounted to 50%.

The process of the clone growth (including the beginning of the stationary phase), incubated after different times of the cell exposure to CHX, corresponds to the date presented in Fig. 2. Thus the phases of the clone cycle depended entirely on the inhibitor concentration and not the time of the drug effect on the cells.

Compared to controls, autogamy occurred with a 2-3 day delay and it was observed only in the cells exposed to CHX at 0.1 $\mu\text{g}/\text{ml}$. Post-autogamous cells were not observed at any of the higher concentrations. The experiment was conducted for 40 days.

The Course of Changes in the Daily Fission Rate of *Euplotes minuta* after their Transfer from a Medium with CHX to a Control Medium

The cells transferred after different time of CHX treatment at 0.1 and 1 $\mu\text{g}/\text{ml}$ to a control medium, reinitiate immediately the fission rate characteristic of untreated cells (Table 5). The cells which were previously exposed to CHX at 2 $\mu\text{g}/\text{ml}$ had a statistically significant reduction in division rate only on the first day in the control medium.

These experiments demonstrated that the time required for reinitiation of the control fission rate of *E. minuta* depends on the CHX concentration employed and not on the time of the cell contact with the inhibitor.

The Measurement of the Macronuclear DNA Content

The contents of the macronuclear DNA (phase G_1) in the control cells and the cells treated for 20 h with 1 $\mu\text{g}/\text{ml}$ CHX were compared.

Since the cell generation time at 1 μg CHX/ml is 15 h, then cells which are in the phase G_1 after 20 h contact with the inhibitor, are these which initiated and completed the synthesis of macronuclear DNA already in the presence of the inhibitor. The average amount of DNA

Table 5

Daily fission rate of CHX-pretreated *Euplotes minuta* in control medium (without CHX)

Time of pretreatment (in days)	Doses of CHX (in $\mu\text{g/ml}$)	Daily fission rate of cells transferred to control medium (days following transfer)		
		I	II	III
1	0	2.04	1.95	1.20
	0.1	2.00	2.04	1.35
	1	1.88	2.14	1.24
2	0	2.02	2.27	1.60
	0.1	2.21	2.24	1.80
	1	2.20	2.60	1.86
5	0	2.13	1.93	1.65
	0.1	1.86	2.55	1.76
	1	1.95	2.40	1.56
5	0	3.00	2.66	—
	1	2.89	2.56	—
	2	2.05	2.60	—
19	0	2.60	2.45	—
	1	2.72	2.23	—
	2	2.20	2.45	—

In the first 3 experiments 20-25 cells from each medium were transferred to fresh control medium. In the two next experiments 4×10 cells from each medium (removed every other day) were transferred to control medium; 2 samples were counted after 24 h, 2 others after 48 h. Every counted cells was removed. Prior to a transfer, cells were rinsed with sea water. "0" — control medium without CHX.

in the macronuclei of CHX-treated and control cells did not show statistically significant differences ($P \geq 95\%$). For the control cells it was 0.0205 ± 0.0071 ($n = 47$) and for the CHX-treated cells, 0.0235 ± 0.006 ($n = 20$) arbitrary units/nucleus. The minimal and maximal recorded values were, respectively 0.012, and 0.034 for control and 0.017 and 0.034 for treated cells.

Discussion

In no case did the addition of CHX bring about a cessation of *Euplotes* fission, nor was there observed a quick reinitiation of the normal fission rate characteristic of control cells. It is evident that CHX does not induce any increased resistance to the drug. These results are different from the data recorded for *Tetrahymena* (Frankel 1970,

Roberts and Orias 1974, Wang and Hooper 1978) and *Chilodonella* (Kiersnowska 1982) cells which manifest quick endogenous recovery in the presence of CHX. The response of *Euplotes* to CHX and AMD (Kiersnowska and Jasińska 1984) is probably similar to that of *Paramecium* to these inhibitors (Suhama and Hanson 1971, Gill and Hanson 1968) and it is a convincing demonstration that the capacity for endogenous recovery is not common to all ciliates.

Ciliates are often employed as bioindicators. Compared to ciliates capable to endogenous recovery, those incapable of recovery are more appropriate to detect the presence of drugs in a medium, since their reaction does not change during the period of drug action, nor is it affected by previous contact of cells of a clone with the drug.

Some experiments were designed to test whether, over a period of several cell generations, in the constant presence of CHX, a gradual increase of the average fission rate occurs as a result of faster division of some cells insensitive to the given dose of inhibitor. As discussed in the introduction, the particular structure and the irregular division of the macronucleus make it possible for a resistant macronuclear variant to occur in a ciliate culture more frequently than in a mammalian cell culture, i.e., more frequently than 1:100 000. This resistance could be the result of an increase in the r-DNA gene count, since a greater number of ribosomes could reduce the net effect of CHX action on ribosomes. Additionally, an increased other genes and their products may also neutralize the action of CHX. If the progeny of such a cell was equally resistant and it divided with the same rate as control cells, then, assuming that the resistant cell appears with the frequency 1:10 000, after 8 days of CHX treatment, sensitive cells would practically be eliminated from a culture containing 2 and 5 μg CHX/ml. Afterwards, resistant cells would outnumber other cells even in 1 μg CHX/ml. The occurrence with this frequency of an even partially resistant variant, i.e., dividing slightly slower than control cells but faster than sensitive cells, would be observed in the 29 day experiment. Nevertheless, no increase in the daily average fission rate indicating such positive selection of a resistant macronuclear variant was ever observed. We feel that if such variants were present, we would have detected them. The concentrations of CHX employed, 1–5 $\mu\text{g}/\text{ml}$, were sufficient to produce variations in the generation times. The macronuclear DNA underwent many rounds of replication and partitioning to daughter cells. The tested cells manifested a considerable variability of macronuclear DNA content and they originated from many different exautogamous lines. The tests were made on both isolates and mass cultures.

The question arises as to why no positive selection for resistant cells was observed. It can be argued that a macronuclear variant may have occurred at a much lower frequency than 1 : 10 000. On the other hand, one may speculate too, that a cell(s) with a higher number of genes, whose product(s) could neutralize the effects of CHX, appeared during the experiments but these quantitative differences were eliminated in subsequent division(s) and/or that regulation of the qualitative gene content of the macronucleus took place.

The regulation of the "quantitative" macronuclear content of *Tetrahymena* and *Paramecium* has been discussed (Doerder 1979, Berger 1979, Cleffmann 1980). In the cultures of these specimens, 2% of the cells with an extremely high or low macronuclear DNA content is observed, but it is reduced during subsequent divisions. For both ciliates different methods of quantitative regulation of their DNA content have been proposed.

Some indirect evidence from genetic experiments on ciliates has allowed speculation about possible mechanism(s) responsible for the "qualitative" gene content of a macronucleus (Preparate and Nanney 1977, Nanney and Preparate 1979, Preer and Preer 1979).

If one assumes that the mechanisms proposed to control "quantitative" or "qualitative" DNA content of *Tetrahymena* or *Paramecium* also operate in the *Euplotes* macronucleus, CHX at least at the doses employed in the present study do not effect these mechanisms. If CHX did inhibit these mechanisms, positive selection of resistant cell lines should have been observed during a long-term experiments.

In *Euplotes minuta* cultures exposed to CHX not only was the cell fission rate reduced also the stationary phase was begun at a lower cell density than in parallel control cultures. This result was not entirely unexpected (see discussion in Kiersnowska and Jasińska 1984) particularly in cells incapable of endogenous recovery from the effects of a drug directly affecting ribosomes.

If the number of ribosomes per cell decreases gradually during the log-phase (Kjelgaard and Gausig 1974) it becomes obvious that at the presence of an inhibitor such as CHX, the number of active ribosomes will be reduced more quickly than in non-treated cells. Thus the stationary phase of the CHX-treated clonal cultures would be achieved after smaller cell fissions number in comparison to the cells growing in control conditions.

The observed fluctuations in the daily fission rate between cycles of autogamy probably result from not only the imperceptible changes in the medium parameters but may also be a consequence of the age of cells in their sexual cycle. It is known that the generation time of

immature, mature and aging ciliates are different (Takagi and Yoshida 1980, Williams 1980, Schwartz and Meister 1975). This change of the cell cycle duration is independent of foods conditions (Grebecki 1961, Legner 1979). The prolongation of the *Euplotes* generation times just after and before the autogamy was noted for the control cells (unpublished dates). In the same periods the decrease of the fission rate at the presence of the CHX (up to 2 $\mu\text{g/ml}$) have been observed too. The effect of some endogenous factors influent on the cell cycle duration was masked only by CHX at 5 $\mu\text{g/ml}$.

The concentrations of CHX employed however, were sufficiently high to inhibit the normal process of autogamy. The present study did not clearly demonstrate whether autogamy took place, or whether the process of the nuclear apparatus reorganization was affected. It has been shown that CHX can bring about the phenomenon of macronuclear retention (Kaczanowski 1981).

Cycloheximide at the concentrations employed did not cause an accumulation of any permanent or temporary injuries. A reduction of the fission rate brought about by its action was completely reversible after the inhibitor had been removed from the medium.

The results presented here support the conclusion that a CHX only slowed the course of the cell cycle, in relation to the rate of the protein synthesis inhibition, but does not provoked any specific modification of the *Euplotes* metabolic pathways towards increase of the cell resistant. It should be also mentioned that in the contrast to *Tetrahymena* and *Chilodonella* the resorption of the nascent cortical structures for daughter cell was not occurred in the CHX treated *Euplotes* (data not presented here).

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Effects of Continuous Presence of Actinomycin D on the Clonal
Growth of *Euplotes minuta*

Mauryla KIERSNOWSKA and Leokadia JASIŃSKA

Institute of Zoology, Warsaw University, P-00-927/1, Warsaw, Poland

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Synopsis. Endogenous recovery may be defined as a total recovery of all cell functions including normal fission rate of all cells in the constant presence of an active drug, which initially could temporarily block cell divisions. From the present study it is deduced that in *Euplotes* a recovery of the daily fission rate in the presence of 0.5-5 µg/ml of actinomycin D (AMD) is not possible. Clones grown in the presence of AMD manifest a decrease of fission rate in relation to the concentration of the drug, without an initial arrest of cell division. This decrease is maintained as long as they are kept in the presence of a given dose of the drug (up to 6 days). The effect of AMD is totally reversible if the drug is removed. Clones growing in the presence of AMD entered the stationary phase after fewer cell generations than the control clones (i.e., non-AMD-treated).

The phenomenon of endogenous recovery (also called recovery or adaptation) is well known in *Tetrahymena* and *Chilodonella*. Generally this phenomenon involves an initial cessation of culture growth followed by resumed divisions of all treated cells, while the active drug is continuously present in the medium. However, it is not yet clear whether the capacity to recover from the effects of many drugs is a common feature of all ciliates. *Tetrahymena* (Frankel 1970, Wang and Hooper 1978) or *Chilodonella* (Kiersnowska 1982) divide at the same rate as controls (i.e., non-treated cells) when they recover from the effects of cycloheximide. But generation times of the recovery of *Tetrahymena* from the actinomycin D effect are prolonged in relation to drug concentrations (Satir 1967). Therefore in the latter case only a partial recovery may take place in contrast to a full recovery from the effect of cycloheximide (see discussion: Heyer and Frankel 1971).

In this communication, the capacity of *Euplotes minuta* (*Hypotricha*) to recover from actinomycin D (AMD) effects is analyzed. To determine the response of *Euplotes*, the fission rates on subsequent days (daily fission rate) of clone growth were tested.

Materials and Methods

Euplotes minuta, strain A-23 (kindly supplied by Dr Heckmann) was grown in synthetic sea water (Kirby 1950), ciliates growing in both mass and clones cultures were always fed the same amount of flagellate — *Dunaliella* sp. Every other day about 2000 cells from mass cultures were replaced to 5 ml of fresh medium, supplied with food to maintain the exponential (log) phase of growth.

Dunaliella was cultured in a medium consisting of 500 ml of synthetic sea water, 20 ml soil extract, 0.5 ml 4% $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$, 1 ml 10% NaNO_3 . The soil extract was obtained from 300 g unfertilized boiled forest soil, filtered and then autoclaved. The medium was added to the flagellate culture every 2–3 days, in 1:1 ratio. *Dunaliella* grew in cyclic light changes i.e., 12 h — 8.5 Lx/12 h — 500 Lx. The density of cells per ml was examined by spectrophotometry with a wavelength light of 665 nm. The used concentrations of actinomycin D (CIBA), an inhibitor of RNA synthesis (Cerami et al. 1967) were prepared 2–3 h prior to the experiment and kept in darkness. In a preliminary test it was demonstrated that concentrations below 5 $\mu\text{g/ml}$ were nonlethal.

Single randomly isolated cells from mass cultures were transferred to depression slides containing 0.75 ml medium without (control) or with a given dose of AMD. In the experiment in which the medium was renewed every 48 h, on the third and fifth day single cells were isolated from these clones where the daily fission rate was closest to the average daily fission rate of all clones in a given series. All clones were grown in darkness to avoid a possible decomposition of AMD in the light. The growth of the control clones kept in darkness was slightly slower than that of clones cultivated in natural light conditions, i.e., the difference was about 0.5 division per day during log phase. The number of cells was scored every 24 h, and daily fission rate was calculated using the formula:

$$f = \frac{\ln N_2 - \ln N_1}{t \ln 2}$$

where: N_1 — cell count at time t_1 ; N_2 — cell count at time t_2 ; $t = t_2 - t_1$.

In a statistical analysis, the standard deviation and the dispersion of all measurements about a weighted mean for all clones growing in the given medium, were calculated (Armitage 1978).

Results

The effects of three AMD concentrations on the daily fission rate of *Euplotes minuta* are presented in Fig. 1. In relation to the concentration employed (0.5, 1 and 5 $\mu\text{g/ml}$) the rate of cell divisions was consi-

derably reduced. The average fission rate for the first 12 h and the next 12 h of the clonal growth in the presence 1 μg AMD per ml were for $n = 20$ respectively 0.47 and 0.6 (data not showed in Fig. 1).

The phase of the most intensive growth for the control clones involves the first 48 h, and during the next three days the decline of the speed of cell multiplication (decelerating phase) is observed. On the 6th day a decrease of the number of cells has been noted (data not showed in Fig. 1), so the stationary phase of these clones must have begun during the 5th day of experiments.

In contrast to the control, daily fission rate of AMD-treated clones remained nearly at a constant level up to 3, 4 and 5 day at the concentrations of 0.5, 1 and 5 $\mu\text{g}/\text{ml}$, respectively. The decrease of celles number occurred on the 6th day (data not shown), regardless of the number of generations yielded in different AMD concentrations employed. It may be suggested that the decelerating phase is shorter (in 0.5 $\mu\text{g}/\text{ml}$) or even omitted (in 1 or 5 $\mu\text{g}/\text{ml}$) and that AMD-treated clones enter the stationary phase at the same period as control clones. It cannot be ruled out, however, that the observed changes of the clonal growth were owing not only to the AMD direct action on *Euplotes*, but also to the secondary drug-effects on the *Dunaliella* serving as food. As a matter of fact, on the third day of drug presence, some non-swimming *Dunaliella* were observed at the bottom of depression slides. Since *Euplotes minuta* does not feed on non-swimming flagellates (Heckmann's pers. comm.), the reduction of food can additionally affect clonal growth. In view of this fact two further experiments were made.

The growth of clones in a control medium containing half the normal amount of *Dunaliella* was tested. The diminution of the amount of food does not change the fission rate of *Euplotes*. In another experiment the daily fission rate of the cells transferred every other day to a fresh AMD medium (1 $\mu\text{g}/\text{ml}$) was examined. The daily fission rate remained on a stable level in a given medium, up to the 6th day of the experiment (Fig. 2).

Additional experiments were carried out in order to test whether the 48 h presence of AMD in the medium brought about any changes in: (1) nutritive conditions or (2) drug activity. The "naive" cells (i.e., those which had no previous contact with AMD) were introduced respectively to: (1) control medium containing *Dunaliella* pretreated with AMD (1 $\mu\text{g}/\text{ml}$) for 48 h, or (2) a 48 hours-old medium with AMD (1 $\mu\text{g}/\text{ml}$) from which all cells were removed. In the first experiment, *Euplotes* divided at the same level as cells fed on flagellates not exposed to AMD pretreatment. In the second experiment the same reduction of the

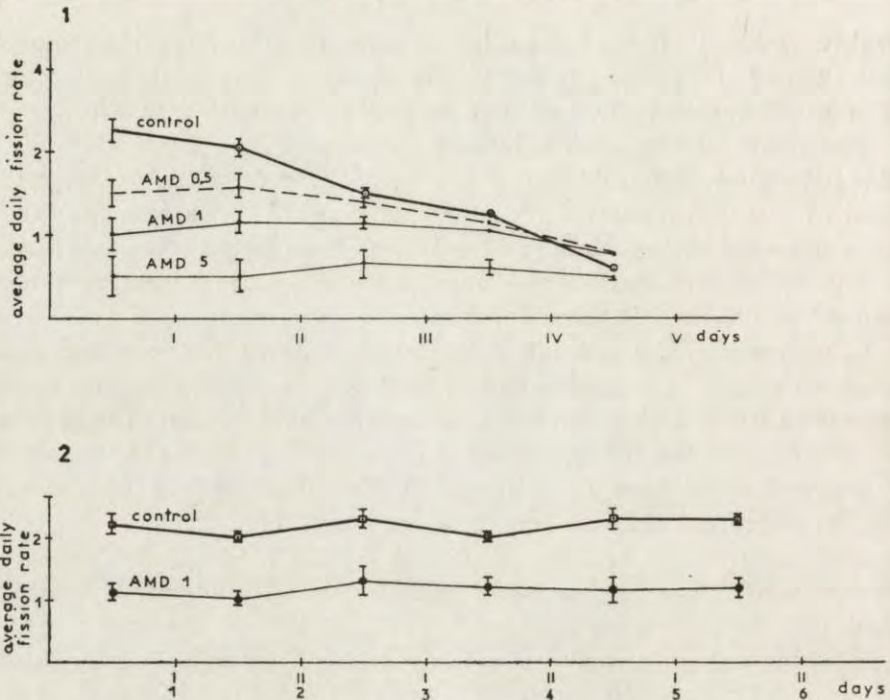


Fig. 1. The effect of actinomycin D (0.5–5 μg per ml) on the *Euplotes minuta* fission rate. The number of tested clones were: 48 in control medium (3 experiments \times 16 clones in each), 45 in 0.5 μg AMD per ml or 1 μg AMD per ml (3 experiments \times 15 clones each) and 30 in 5 μg AMD per ml (2 experiments \times 15 clones in each)

Fig. 2. The effect of actinomycin D (1 μg per ml) on the fission rate of *Euplotes minuta* growing in the medium renewed every 48 h. On the first day 30 and 15 clones were inoculated in the medium without and with AMD, respectively; on the third and fifth days 15 cells from each medium were transferred to a fresh medium

fission rate was observed as in the “naive” cells transferred to a fresh medium containing 1 $\mu\text{g}/\text{ml}$ AMD per ml.

These data are taken as an evidence that the AMD effect on *Euplotes* resulted exclusively from a direct action of AMD on ciliates, without the effect of starvation.

In order to check whether AMD brought an irreversible damage to *Euplotes*, cells growing 48 h in the presence of 1 μg AMD per ml were again replaced in a control medium, and then their daily fission rate was tested. AMD-pretreated cells ($n = 40$) and control cells ($n = 20$) divided at the same level. Even on the first day after the transfer the daily fission rate was statistically identical ($P \geq 95$) with that of the control.

Discussion

Results obtained in the present study demonstrate that *Euplotes minuta* is not capable of endogenous recovery from the effects of AMD, since the average fission rate in a given concentration remains at the same reduced level from the first to the 6th day of the inhibitor action. The fact that the studies of AMD effects were carried out on over a hundred isolated cells allows us to conclude that an addition of the drug to a logarithmically growing mass cultures of *Euplotes minuta* will not result in an immediate cessation of the culture growth. Thus the response of *Euplotes* is quite different from that of *Tetrahymena*. Therefore it may be suggested that the endogenous recovery is not a common feature of all ciliates.

It is possible that a similar response to that of *Euplotes* is manifested by *Paramecium* whose daily fission rate in the presence of non-lethal, very low concentrations of CHX (Suhama and Hansen 1971) and AMD (Gill and Hanson 1968) is subjected to small variations. However, the cited studies were not focussed on the problem of endogenous recovery, so the available data are insufficient to conclude whether *Paramecium* is capable of recovery or not. Khan and Nassren (1981) observed some reduction of the fission rate of hypotrichous cells-*Oxytricha fallax* exposed to Pb.-nitrate. There are no data whether this drug brought about an initial inhibition of cell divisions, and therefore the kinetics of reaction of *Oxytricha* to this inhibition is not fully documented.

The concentrations of AMD employed in the present study, do not cause any permanent damage to *Euplotes*, since AMD treated cells replaced to a control medium immediately started to divide, just like cells always kept in a control medium.

The effect of AMD on both the decelerating and stationary phase of *Euplotes* cultures is not quite surprising. Satir (1967) observed that *Tetrahymena* grown in a continuous presence of AMD entered the stationary phase after a lower number of generations than those kept in a control medium. The increase of amount of *Tetrahymena's* RNA is linear during the logarithmic phase of the culture growth, so that the doubling of RNA amount is slower than that of the cell number. If 80% of the total cellular RNA represents the r-RNA, the number of ribosomes in a cell gradually decreases with subsequent divisions. It must be also mentioned that RNA synthesis is arrested during stationary phases (Cameron and Guile 1965). In eukaryotic cells, low doses of AMD can block the synthesis of preribosomal RNA, while the non-

ribosomal transcription is relatively little affected (Puvion-Dutilleul and Bachellerie 1970). Even short-term action of AMD (low doses) brings about substantial modification of the ribosomal transcription. Since both, the course of cell cycle and the course of culture growth are controlled by the number of active genes, it is possible that the decrease of the number of ribosomes per cell below the minimum level will occur more quickly in the presence of a such metabolic inhibitor as AMD than in cells growing under control conditions.

The ability of *Euplotes* to maintain the fission rate on the same level at a given AMD concentration can be explained by: (1) non-saturation of all binding AMD sites, since every DNA sequence can be present in the macronucleus of *Hypotricha* in about 1000 copies (Lauth et al. 1976), and r-RNA genes are repeated about 100 times more frequently than other genes (Swanson et al. 1980); and/or (2) the establishment of some balance between inactivation and some repair processes.

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Production of *Amoeba proteus* Clones with Increased Nuclear DNA Content

S. Yu. AFON'KIN

Laboratory of Cytology of Unicellular Organisms, Institute of Cytology of the Academy of Sciences of the USSR, 4 Tikhoretsky prosp., Leningrad 194064, USSR

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Synopsis. By injecting colchicine into dividing cells of three *A. proteus* strains, in 38% of the cases karyokinesis was blocked. Cells after C-mitosis yielded clones (col-clones). The DNA content in the nuclei of col-clones was reliably larger than in the control. A mass culture was grown from one col-clone (strain B). Dividing cells of the mass culture were injected colchicine for the second time. As a result cells with double-blocked mitosis were obtained (2col-cells). From one of such cells a mass culture was also grown. The evidence of the DNA content in the nuclei is in agreement with the hypothesis of a two and four-fold increase of the latter in col- and 2col-clones. Accordingly, the area of metaphase plates increases two- and fourfold and the nuclear area increases 1.6- and 2.7 times as compared to the control. The mean generation time remains unchanged. The cell volume in col- and 2col-clones increases 1.2- and 1.7 times, respectively, which indicates some alterations in the nucleo-plasmic relationships. Since the precise number of chromosomes in *A. proteus* is hard to count and the original ploidy level is not known, the produced col- and 2col-clones may be assumed as tetraploid and octoploid with a reserve. The obtained clones may be used for elucidation of the gene dose effect on the phenotypic characters of amoebae and also in experiments in production of nucleo-cytoplasmic "hybrids". In hybrids produced by transplantation of normal nucleus into the enucleated 2col-cytoplasm, and by transplantation of 2col-nucleus into the enucleated normal cytoplasm, it is proved that the increased DNA content is transferred with the nucleus.

Polyploidy plays a large part in the evolution of many cell types, protozoan cells among them (Poljansky and Raikov 1960, 1977). Unicellular organisms are suitable objects for studying the influence of polyploidy on the cell. Induction of C-mitosis by microtubular poisons

has been extensively used to produce polyploid cells in experimental conditions. By injecting colchicine solution into dividing *Amoeba sphaeronucleus* cells Commandon et de Fonbrune (1942 a, b) managed to block mitosis. From the operated amoebae they succeeded to obtain progeny with enlarged cell and nuclear volumes. The experimentally produced clones were successfully cultivated over prolonged periods. The authors centered their attention on the analysis of nuclear-cytoplasmic relationships in normal and experimentally obtained amoebae, but made no detailed study of the latter. Specifically, they did not measure the nuclear DNA content. Clones of *A. sphaeronucleus* with enlarged nuclei Commandon et de Fonbrune had produced, were not used later on by other investigators.

At present another species of free-living amoebae — *Amoeba proteus* — is more often used for cytological studies (Yudin 1982). The paper describes the production of *A. proteus* clones with increased nuclear DNA content through blocking mitosis with colchicine.

Materials and Methods

Amoeba strains. Three *Amoeba proteus* strains (B, C, L) were used for the experiments. The C and L strains were isolated from waterbodies in the Leningrad region, the B strain was received from Danielli's laboratory (Great Britain). As has been recently shown (Afon'kin 1983) the DNA nuclear content did not differ significantly in the three strains.

Amoebae were cultivated at 25°C according to Prescott and Carrier (1964) and fed on *Tetrahymena pyriformis*. Culture medium was changed daily, food was given every other day.

Colchicine injections. Since the presence of colchicine in culture medium does not affect *A. proteus* (King and Beams 1940), colchicine solution was injected directly into dividing cells. The existence of a definite correlation between the morphology of the dividing cell ("division sphere") and its mitotic stage (Ord 1970 a, b) enabled us to select from mass culture those amoebae which had passed prophase but did not start cytokinesis. Injections were carried out with the aid of MM-I micromanipulator using agar slide (Jeon and Lorch 1968).

To introduce colchicine solution into amoebae, glass micropipettes with tips of about 6 μm inner diameter were made with the aid of a "Carl Zeiss" microforge. Each micropipette was measured under a microscope by means of an eyepiece micrometer. The micropipette was filled with paraffin oil and thereafter with 3% colchicine solution (Fluka AG, Switzerland). The volume of this solution was calculated so that its final concentration within the cell was about 0.01%. The cell volume of *A. proteus* was assumed to range from $1.5 \times 10^{-8} \text{ mm}^3$ to $2.7 \times 10^{-8} \text{ mm}^3$ (Ord 1968 b).

Cells and clones obtained by injection of culture medium into dividing cells were used as a control. After operation cells were placed in microaquaria, and

after 24 h the number of nuclei was determined in those cells which underwent no division. Cells that turned to be mononuclear were cloned.

Isolation and measurement of nuclei. Nuclei were isolated by destroying cells in culture medium by means of a micropipette. Thereupon a nucleus in a minimum volume of culture medium was transferred onto a slide. When drying the nucleus stuck to the slide surface.

The nuclei contours were sketched using a camera lucida at the magnification 20×40 . The nuclear area was determined with the aid of a planimeter. The accuracy of measurement of the same contour was 1.3%. The normal *A. proteus* nucleus has the shape of a disc, the ratio between its height and diameter being 1:4 (Davson et al. 1937, Roth et al. 1960). Therefore the nucleus always falls on the slide with one of its flat sides, and its outlines make a circle.

When comparing the nuclear areas of cells' original and experimentally obtained clones, the amoebae were synchronized by selection of "division spheres" from mass cultures. Dividing amoebae were placed in a medium with food, and samples of 20-30 cells were taken over definite intervals up to the onset of the next division. Selection from mass culture of about 100 dividing amoebae and isolation of nuclei from 20-30 cells required from 15 to 20 min. Consequently the maximum age difference between cells in thus synchronized samples was about 40 min.

Measurements of nuclear DNA content. The relative DNA content in isolated nuclei was estimated cytofluorimetrically by registering fluorescence of the dye Auramine 00 bound to DNA. Every five nuclei transferred onto a slide were fixed with methanol.

To avoid systematic errors caused by drift in the instrument sensitivity in time the intensity of nuclei fluorescence was expressed in arbitrary units. The fluorescence intensity of rat hepatocyte tetraploid nuclei (G_2 phase) was assumed for 1 arbitrary unit.

The technique of staining and fluorescence measurement has been previously described in detail (Makhlin et al. 1979 a). The DNA nuclear content was determined in synchronized cells. The earliest time for sampling was 1 h after the completion of cytokinesis, when the envelopes of daughter nuclei were fully formed and the latter could be isolated from cells.

In the case of small clones, when we could not isolate a sufficient number of synchronously dividing amoebae, the DNA content was measured in partly synchronized samples. To do this, a group of cells was isolated randomly from the clone and cultivated in a medium with food for 24 h. Cells that had underwent division were rejected. Thus the remaining cells had a minimal age of 24 h.

To render the comparison of the DNA content for different samples more dependable, we correlated data obtained for nuclei fixed on the same slide and thus stained simultaneously. It is known that in measurements of the DNA content in the nuclei of the same age from amoebae of one strain fixed on different preparations and stained at different time the mean DNA content may differ significantly. Moreover, such discrepancies are observed occasionally between samples fixed on the same slide (Makhlin et al. 1979 a, Afon'kin 1983).

Measurements of amoeba metaphase plates. For measuring metaphase plates of amoebae from original and experimentally obtained clones dividing cells from mass cultures were treated on a slide with 45% hot acetic acid. As a result, the cells were flattened on the slide. Thirty seconds after the acid was drawn off, the preparations were dried and stained with Auramine 00 by

the method adopted as standard for staining isolated nuclei. Fluorescing metaphase plates were photographed using a RF-3 film sensitive to green rays at the magnification 3×90 . The plate diameter was determined in the photographs (accuracy of a single measurement — $0.1 \mu\text{m}$) and then mean square values were calculated.

Measurements of cell volume. The amoeba was placed in a roto-compressor (Fokin and Ossipov 1975) and pressed down to the same thickness and then cell contours were sketched using a camera lucida at the magnification 10×15 . The cell area was determined with the aid of a planimeter. Since the thickness of the flattened cells was the same within the instrument precision, the cell areas correlated with the cell volumes. For such measurements synchronized cells were used. The accuracy of the cell volume determination derived from repeated measurements of the same cell, was 1.2%.

Evaluation of mean generation time. Mean generation time was determined by two methods: (1) Samples of 50 cells were selected randomly from clones under investigation. The cells were cultured individually for 8–10 days. The formula

$$n = \frac{\lg N}{\lg 2}$$

(Nachtwey and Cameron 1978), where N is the mean number of cells in a subclone, and n is the mean number of doublings of a cell population in a subclone, was applied to calculate the mean generation time. (2) We registered the moment of time when 50 out of 100 synchronized amoebae had completed their division.

In the first case subclones with a low number of cells (less than 4 after 8–10 days) were rejected, and in the second all binucleate cells, since we did not know when their nuclear division occurred.

Producing of nuclear-cytoplasmic "hybrids". Nuclear-cytoplasmic "hybrids" were produced according to Commandon et de Fonbrune (1939). For details see Yudin (1974).

Results

Producing Clones with Double Nuclear DNA Content

The fate of amoebae injected with colchicine solution during division is different (Table 1). Few operated cells died within 24 h. The proportion of dying cells is similar after injections of both colchicine and culture solution, their death being probably due to irreversible mechanical injuries inflicted on the cells by the operation. About one third of the cells complete their division after colchicine injections. In the control sample two thirds of the operated cells proceed with their division. This may be explained by the fact that it is impossible to identify the mitotic phase when selecting dividing cells from a mass culture. Not unfrequently, therefore, chromosomes have had time to reach the oppo-

Table 1

The fate of strain B amoebae 24 h after colchicine injection during division

Experimental variant	Number of cells injected	Number of destroyed cells	Number of dividing cells	Number of cells non-divided after the operation			
				mono-nucleate	bi-nucleate	tri-nucleate	tetra-nucleate
Colchicine injection Prescott medium	250	25	66 (29%)	85 (38%)	67 (30%)	3 (1%)	4 (2%)
injection	61	9	32 (62%)	6 (11%)	14 (27%)	—	—

The percent was determined from the total number of operated cells minus destroyed cells.

site poles by the beginning of the operation. This also might account for a considerable number of cells which become binucleate after the operation. Thirty eight percent of amoebae underwent no division and had only one nucleus. Such cells occur also in the control sample, which implies that even a purely mechanical injury can disturb the nuclear division. However, the percent of mononucleate amoebae in the controls is lower by two thirds than in the experimental cells, and, therefore, colchicine injections increase significantly the percent of cells which have not completed the nuclear division.

The three- and four-nucleate cells that occur after the operation are likely to originate from occasional binucleate cells in amoebae mass cultures (Sopina et al. 1982). Thirty five out of 85 mononucleate (after colchicine injections) cells of B strain were accidentally lost, and the remaining 50 cells yielded only 23 clones henceforth designated Bcol (col for colchicine). The rest of the cells (27) appeared to be non-viable. Four Ccol clones and three Lcol clones were obtained in the same way from C and L strain amoebae.

One month later, when the number of amoebae in the clones amounted to some hundreds, the nuclear DNA content was measured in partly synchronized samples in clones Lcol, Ccol, Bcol and in the control (Table 2). The average nuclear DNA content of the original B, C and L amoebae fixed on the same preparation did not show significant differences, while the nuclear DNA content of all col-clones was 1.5-2.6 times higher than that of the control. So by cultivating amoebae cells, which had one nucleus after they had received colchicine injection during division one may obtain clones with an increased nuclear DNA content. Such clones were grown from amoebae of all the three strains (B, C, L).

Table 2
Nuclear DNA content in partly synchronized cells from col-
clones one month after their production

Clone	Number of measured nuclei	DNA content $\bar{x} \pm S_{\bar{x}}$ arb. units
B	58	2.1 ± 0.1
Bcol-1	68	4.3 ± 0.1
Bcol-2	67	4.6 ± 0.1
Bcol-3	49	4.1 ± 0.1
Bcol-4	51	4.6 ± 0.2
Bcol-5	50	5.5 ± 0.2
Bcol-6	36	3.6 ± 0.2
Bcol-7	60	4.3 ± 0.1
Bcol-8	52	5.1 ± 0.1
C	30	2.2 ± 0.1
Ccol-1	29	4.2 ± 0.1
Ccol-2	28	4.0 ± 0.1
Ccol-3	29	3.9 ± 0.1
Ccol-4	28	4.0 ± 0.1
L	27	2.2 ± 0.1
Lcol-1	23	3.2 ± 0.1
Lcol-2	28	3.9 ± 0.1
Lcol-3	30	3.5 ± 0.1

Some values of the nuclear DNA content in partly synchronized samples of col-clones differ significantly (Table 2). It is most probable that the variations result from inaccurate cell synchronization. This means that samples under comparison may differ in the percentage of cells which have synthesized a definite amount of DNA by the time of nuclei isolation. This suggestion is supported in part by the fact that neither minimal (3.6 arb. units), nor maximal (5.5 arb. units) values of the nuclear DNA content of Bcol amoebae may be discarded as not belonging to the given statistical sample.

To estimate more precisely the amount of nuclear DNA in col-clones and to show that differences between the mean values given in Table 2 are due to some inaccuracy of amoebae synchronization, a mass culture of Bcol-6 cells was grown, i.e., from the clone with the minimal value of DNA content (see Table 2), whereupon the DNA content was determined in the nuclei of synchronized cells from this culture.

The mean generation time for the original culture and Bcol-6 clone was previously shown to be similar (Table 3). It is noteworthy that in our experiments it was somewhat higher than in the earlier ones (Sopina 1976).

Synchronously dividing amoebae, after the completion of their cyto-

Table 3
 Mean generation time in Bcol-6 and B amoebae determined by counting the number of cells in sub-clones after 8 to 10 days cultivation

Experiment	Mean generation time (h)	
	Bcol-6	B
1	66.5±0.5	68.8±0.7
2	59.0±0.4	60.3±0.6
3	58.2±1.2	56.8±0.5
4	74.8±1.0	76.7±1.3

kinesis, were cultured in a medium with food for 1, 5, 24, 48 and 72 h. By the onset of nuclei isolation 80% of 72 h cells in the control and 52% of 72 h cells from Bcol-6 clone had divided, and nuclei were isolated only from the remaining amoebae (still undivided). The nuclear DNA content in the cells of Bcol-6 clone was higher than that in the nuclei of the original strain 2.0, 1.8, 1.9, 1.7 and 1.9 times respectively (Fig. 1).

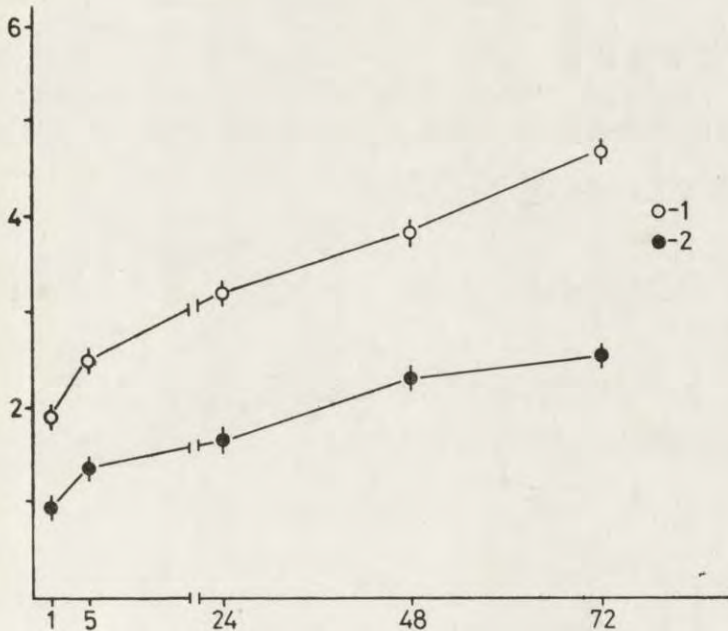


Fig. 1. Increase in the nuclear DNA content during the cell cycle of Bcol-6 (1) and B (2) amoebae. *Abcissa*: time in hours from the end of division to the moment of nuclei isolation, *ordinate*: DNA content in arb. units. Here and henceforth vertical dashes designate the 95% confidence intervals. The number of nuclei in each sample is about 35

DNA synthesis in *A. proteus* is known to start immediately after cell division is completed (Ord 1968 a), i.e., this amoeba lacks G_1 period. There is no evidence as yet that the *A. proteus* nuclei contain the same amount of DNA just before mitosis. Since it is known that the DNA content in the *A. proteus* nucleus is influenced by feeding conditions (Makhlin et al. 1979 b), it would be reasonable to compare the DNA amount in the nuclei of different clones during its synthesis as early as possible, i.e., in "one-hour" nuclei (Afon'kin 1983).

Using the confidence intervals for sample means of 1 h nuclei from Bcol-6 and B amoebae, we calculated minimum and maximum ratios of general means. These ratios were found to be 1.90 and 2.26. This is consistent with the hypothesis that the nuclear DNA content in Bcol-6 clone is doubled as compared with the original B strain.

It is known that the nuclear DNA content over the *A. proteus* cell cycle increases more than twofold (Makhlin et al. 1979 a). This is also characteristic of cells from Bcol-6 clone. The DNA content in 72 h nuclei in this clone exceeds 2.4 times the 1 h level. Seventy two hours after division the nuclear DNA of the original strain is 2.7 fold over the 1 h level. However, these two figures cannot be directly compared since the ratios of the DNA content at the beginning and at the end of a cell cycle may vary from sample to sample even within one strain (Makhlin et al. 1979 a).

On the average, throughout the cell cycle the nuclear area in Bcol-6 clone is 1.6 times larger than in the original strain B (Fig. 2) Upon

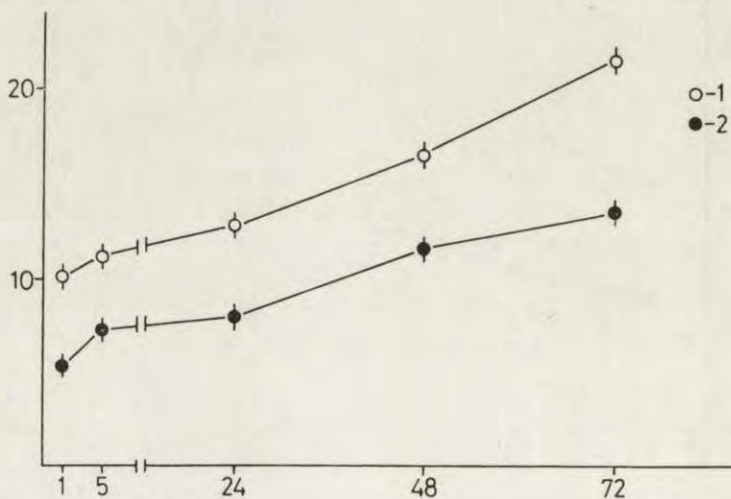


Fig. 2. Increase in the nuclear area during the cell cycle of Bcol-6 (1) and B (2) amoebae. *Abcissa*: time in hours from the end of division to the nuclei isolation, *ordinate*: nuclear area in arb. units (1 arb. unit — $200 \mu\text{m}^2$)

assumption that the *A. proteus* nucleus grows in volume but retains its geometrical similarity, this should indicate a twofold increase of the nucleus volume in Bcol-6 clone as compared with the norm. Theoretically, when the nuclear volume is doubled, the nuclear area should increase 1.58 times.

The increase of the nuclear DNA content during the amoebae cell cycle of Bcol-6 clone and B strain is accompanied by an increase of their nuclear area (Fig. 1, 2). But the correlation coefficient value between these characteristics within synchronized samples (the same nuclei were used to measure the DNA content and the nuclear area) is not always statistically reliable. Thus for 24 h nuclei of B strain the correlation coefficient was found to be $+ 0.41 \pm 0.14$; for 1 h nuclei of Bcol-6 clone it was $+ 0.70 \pm 0.12$; for 5 h nuclei $+ 0.46 \pm 0.15$. In the remaining seven instances the values were not significant.

The number of chromosomes in the *A. proteus* nucleus is immensely high, reaching some hundreds (Liesche 1938). Therefore it cannot be determined precisely in metaphase. That was the reason why we measured only the diameters of metaphase plates. The mean square values of the metaphase plate diameters of dividing cells from clone Bcol-6 were significantly higher than in the control (Table 4). The

Table 4
Size of chromosomal plates in Bcol-6 and B amoebae

Clone	Mitotic stage	Number of measurements	Plate diameter $\bar{x} \pm S_{\bar{x}}$ (μm)
Bcol-6	Metaphase	19	21.3 ± 0.4
	Early anaphase	35	14.0 ± 0.2
B	Metaphase	11	16.0 ± 0.5
	Early anaphase	54	9.5 ± 0.2

\bar{x} — mean square value.

$S_{\bar{x}}$ — mean error.

metaphase plate diameter of cells from Bcol-6 was 1.33 times greater than in the control (Pl. I a, b) while the diameter of early anaphase plates in this clone exceeded 1.47 times that of the control (Pl. I c, d). The values are in good consistency with the theoretical prediction (1.41 times) assuming a twofold increase of the plate area.

It must be noted that out of 185 randomly selected dividing cells from clone Bcol-6 and B strain only 30 cells (16.2%) were metaphasic,

the rest of the cells being in anaphase. This supports our suggestion that not all dividing cells selected from mass culture were in metaphase and that is why only a small portion of operated cells appeared to be mononucleate after colchicine injection (Table 1).

Producing Clones with Fourfold Nuclear DNA Content

To obtain such clones colchicine solution was injected into dividing cells from clone Bcol-6. A total of 185 cells were operated. Fourty four cells died after the operation. Eight operated cells divided, 92 cells were found to be binucleate, and 41 were mononucleate. Twelve mononucleate cells were irreversibly injured during examination, and 29 yielded 2 clones (7% of the total number of mononucleate cells). Thus the percent is the significantly lower than that in the control (46% in strain B).

A mass culture was produced only for one clone, which will be designated as B2col-1 (2col-twofold colchicine injection into the cell). The rate of increase in the number of cells in the subclones B2col-1 is reduced. Only in 40 to 45% of the subclones the number of amoebae by the 10-14 day is more than four cells, while in the control (strain B) this value makes 98-100%.

This might be explained both by the increase of mean generation time of amoebae from clone B2col-1 and by the reduction of survival probability of these cells. It is obvious that data on the cell number in subclones by themselves are not sufficient to choose between the two interpretations. Therefore, the mean generation time of cells from clone B2col-1 was defined by determining the time of division for 50% of the amoebae synchronized during the previous division. Three tests were made. Not in a single case have we found statistically significant differences between the mean generation time of amoebae from clone B2col-1 and that of strain B amoebae (Fig. 3). Consequently, the reduced rate of increase of the cell number in clone B2col-1 results from a greater death rate. It seems that amoebae from clone B2col-1 much more frequently fail to accomplish a successful mitosis.

To evaluate the DNA content in clone B2col-1, samples of amoebae from clone B2col-1, Bcol-6 and strain B synchronized according to the previous division were taken. The ratio between the general means of the DNA content in 1 h nuclei of the amoebae from B2col-1 and Bcol-6 was found to range from 2.01 to 1.75 (the significance level — 0.05). This is consistent with the hypothesis of twofold increase in the nuclear DNA content of cells from B2col-1, as compared to Bcol-6 amoebae (Fig. 4).

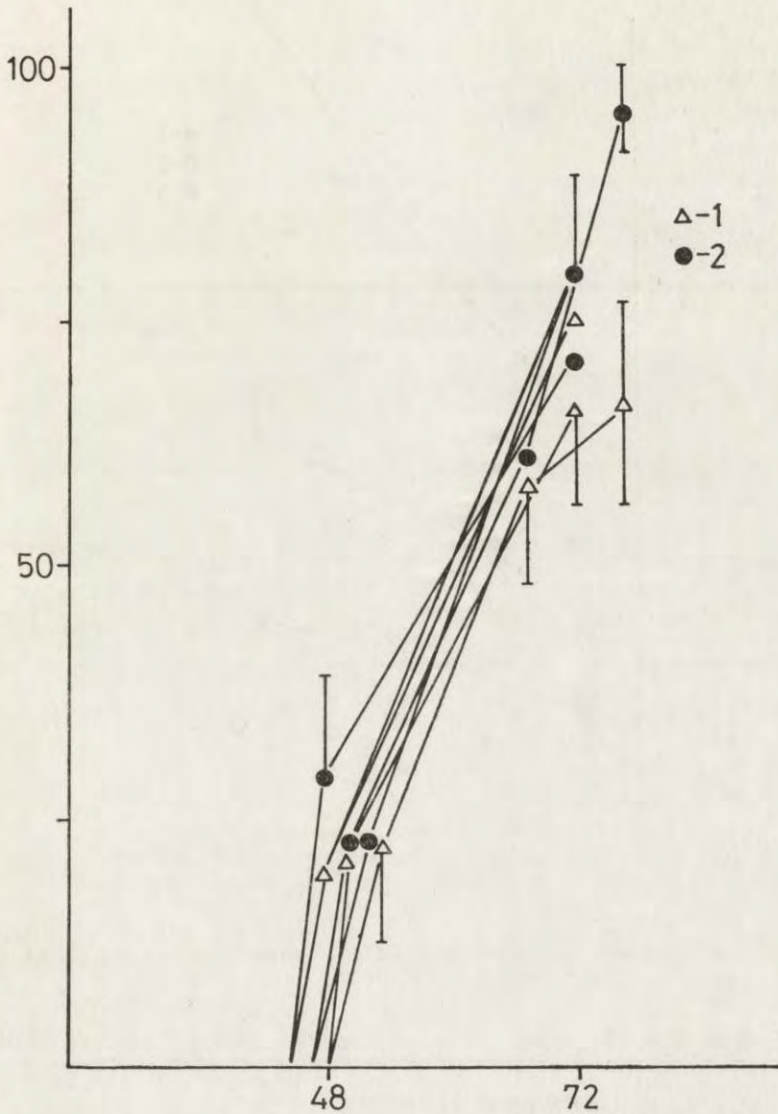


Fig. 3. Accumulated fractions of dividing B2col-1 (1) and B (2) amoebae synchronized by the previous division. *Abscissa*: time in hours, *ordinate*: percentage of dividing cells (%)

This value for the pair Bcol-6 and strain B is within the range of 2.25-1.60. Similarly to cells from Bcol-6 and strain B, in the cell cycle of amoeba from B2col-1 we observe a more than twofold increase in the 1 h content of nuclear DNA. By 50th h the DNA content in the nuclei of the sample under investigation 2.5 times exceeds that of 1 h nuclei.

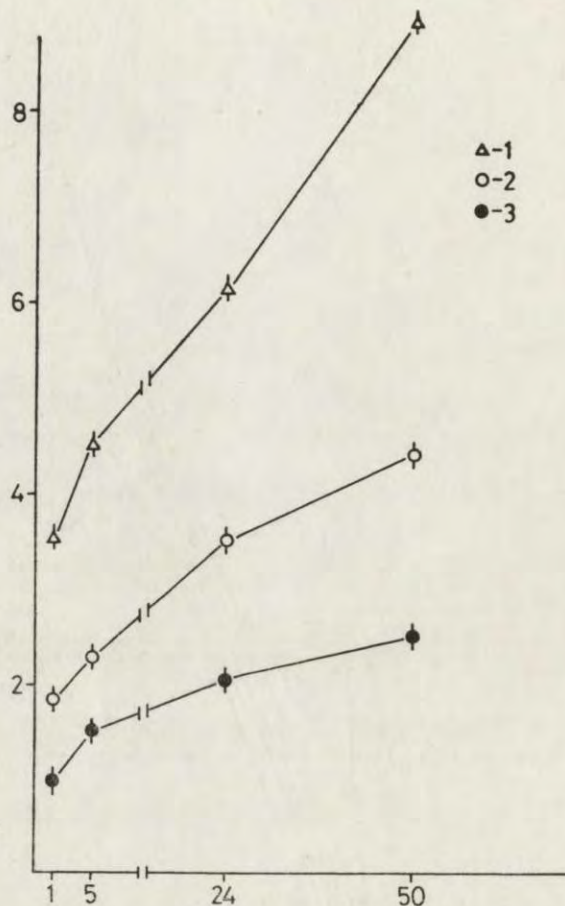


Fig. 4. Increase in the nuclear DNA content during the cell cycle of B2col-1 (1), Bcol-6 (2) and B (3) amoebae. *Abscissa*: time in hours from the end of cell division, *ordinate*: DNA content in arb. units

The mean square value for the metaphase plate diameter of dividing cells from clone B2col-1 is $35.7 \pm 1.0 \mu\text{m}$ (8 plates) which exceeds 1.67 times that for cells from Bcol-6 (Pl. I e).

The nuclear area of the cell from clone B2col-1 is on the average 2.7 times greater than that of strain B amoebae (Fig. 5), the theoretically expected value of the increase of the nuclear area with a fourfold enlargement of the nuclear volume being 2.5.

In clones B2col-1 and Bcol-6, cell volumes were also measured. It was found out that on the average the cell volume in B2col-1 amoebae is 1.4 times over that of Bcol-6 cells, while the cell volume of the latter 1.2 times exceeds the control values (Fig. 6).

The character of "increased nuclear DNA content" in *A. proteus*

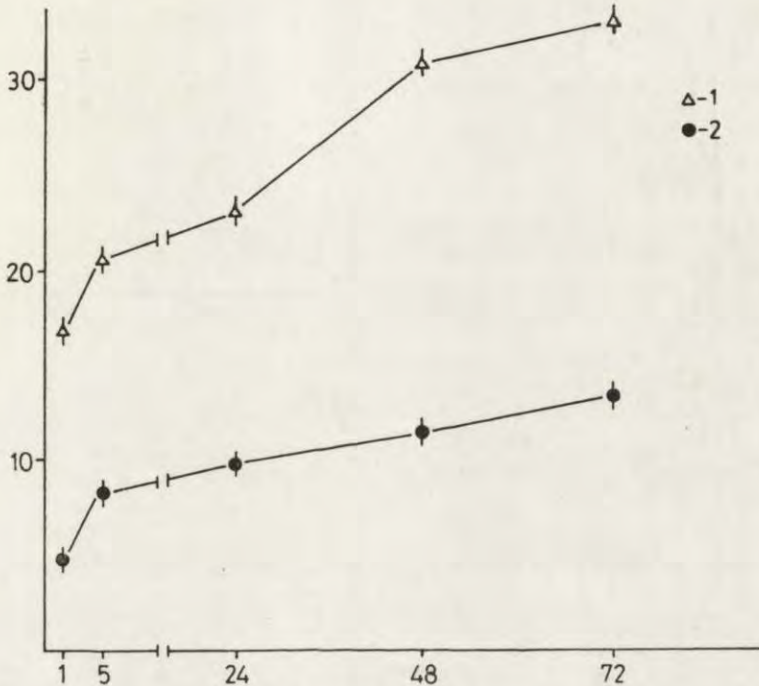


Fig. 5. Increase in the nuclear area of B2col-1 (1) and B (2) amoebae during cell cycle. *Abscissa*: time in hours from the end of cell division, *ordinate*: nuclear area in arb. units

is hereditary. This fact is supported by a prolonged (over many months) cultivation of col-clones prior to the DNA content determination (Fig. 1, 4). This character can be transferred with the cell nucleus when nuclear-cytoplasmic "hybrids" are produced. As a result of transplantation of the nucleus from B amoeba into the enucleated cytoplasm of Bcol-6 cell we obtained "hybrids" $B_nB_{col-6}_c$ (n — nucleus, c — cytoplasm). Reciprocal "hybrids" $B_{col-6}_nB_c$ were also produced. One month after the number of progeny of operated cells amounted to some hundreds, the nuclear DNA content was measured in partly synchronized samples of three randomly selected clones of each type and in the control. Determinations were repeated twice in each clone over the interval of 2 to 7 days. The result (Table 5) shows the character of "increased DNA content" is transferred with the nucleus. These experiments have also shown a complete transplantation compatibility (Makhlin 1981) between the amoebae with increased DNA content and the original B amoebae.

It should be stressed, however, that after a prolonged cultivation in some of the col-clones the average DNA content in samples was

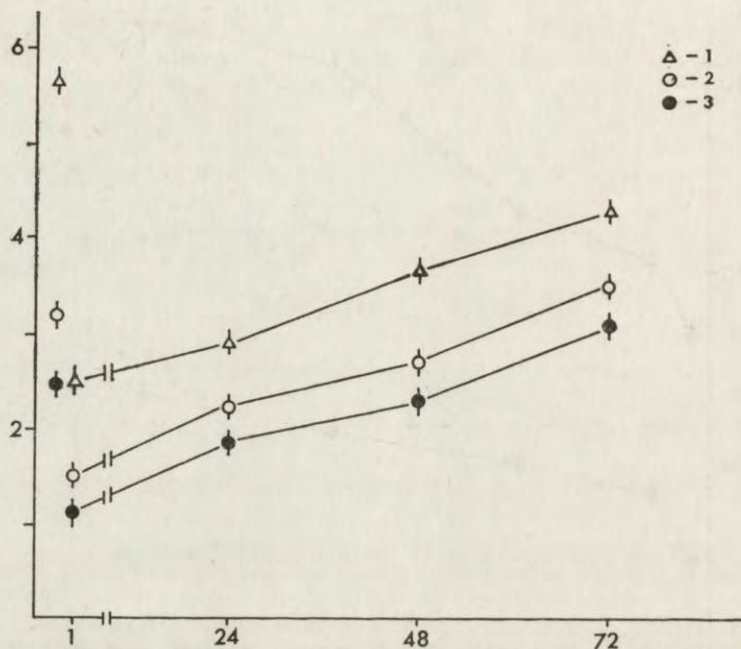


Fig. 6. Increase in the cell volume of B2col-1 (1), Bcol-6 (2) and strain B (3) amoebae in the cell cycle. *Abscissa*: time in hours from the end of cell division, *ordinate*: cell volume in arb. units (1 arb. unit — $1 \times 10^6 \mu\text{m}^3$). Utmost left points — the volume of dividing cells

Table 5

Nuclear DNA content in partly synchronized amoebae grown from nuclear-cytoplasmic "hybrids" $B_nBcol-6_c$ and $Bcol-6_nB_c$

Clone	Number of measured nuclei	DNA content $\bar{x} \pm S_{\bar{x}}$ arb. units
B		
(control)	60	2.5 ± 0.1
$B_nBcol-6_c-1$	64	2.5 ± 0.1
$B_nBcol-6_c-2$	70	2.5 ± 0.1
$B_nBcol-6_c-3$	60	2.5 ± 0.1
Bcol-6		
(control)	60	5.5 ± 0.5
$Bcol-6_nB_c-1$	44	5.5 ± 0.1
$Bcol-6_nB_c-2$	45	5.5 ± 0.1
$Bcol-6_nB_c-3$	43	5.6 ± 0.1

\bar{x} represents an averaged result obtained from two independently measured nuclei samples.

changed due to the appearance of amoebae with the DNA amount not typical of the clone. This phenomenon will be considered in detail elsewhere.

Discussion

Very few protozoan species were treated with colchicine for producing polyploid clones. The cells of a colonial flagellate *Gonium pectonale* (Shyam and Sarma 1976), chlamydomonads (Bell 1955, Wetherell and Krauss 1956), trichomonads (Samuels 1949) were treated with aqueous colchicine solutions. The cells of *Chlamydomonas eugametos* were grown on agar containing 0.2% colchicine (Buffaloe 1959). In all these cases, however, it was mass cultures that were treated with colchicine without subsequent cloning. Therefore these experiments were hard to interpret.

Much more clear-cut evidence can be obtained from experiments with single cells. If in this case colchicine dissolved in a culture medium did not affect the cells (King and Beams 1940, Injeyan et al. 1979) microinjection of the drug into a cell was commonly administered. Thus Commandon et de Fonbrune (1942 a, b) injected colchicine solution into *Amoeba sphaeronucleus* (*Thecamoeba sphaeronucleolus*) dividing cells and obtained clones with the nucleus and cytoplasm volumes twice as large as those in amoebae of the original strain. Until the present time the experiments by Commandon et de Fonbrune have been the only example of producing amoebae clones with enlarged cells and nuclei by means of colchicine injection¹.

In our case we injected colchicine solution into dividing cells of *A. proteus*, a species of free-living amoebae now commonly used in cytological experiments (Jeon 1973). From cells in which karyokinesis was blocked as a result of the operation we grew clones that differed in some characters from the original culture. In clones Bcol-6 and B2col-1 the nuclear area was 1.6 times and 2.7 times greater, respectively, as compared to normal cells. This is ample evidence that changes in the nuclear volume correlate precisely with those in cell ploidy (see Hesin 1967). With a twofold increase of the nuclear volume the

¹ Amoebae clones with nuclei larger and smaller than in normal cells were also obtained by Ord (1970 a, b, 1973). She treated cells with mutagene N-methyl-N-nitrosourethane. It is not unlikely that the DNA content in mutant clones was altered. However, it remains but a suggestion. The genetic nature of the variation is still uncertain.

nuclear area increases 1.6 times (Benjuš 1967). Therefore, the increase of the nuclear area of Bcol-6 amoebae by 1.6 times seems to testify to the duplication of the nuclear volume. As for B2col-1 amoebae, the volume of their nuclei must increase 4.4 times. Accordingly, the area of metaphase plate in Bcol-6 and B2col-1 is 2 and 4 times over that of the original strain. Our data on the nuclear DNA content are consistent with the hypothesis of duplication of the DNA amount in Bcol-6 and of its fourfold increase in B2col-1. All this allows to suggest that in the nuclei of Bcol-6 and B2col-1 amoebae, as a result of colchicine treatment, the chromosome set increases 2 and 4 times.

However, the size of metaphase plate, the nuclear volume, and the DNA nuclear content are but indirect proofs of polyploidy (Nagl 1978). It is known that colchicine affects not only the spindle macrotubules, but also the cytoskeleton. This may provoke the endocycle onset without doubling the number of chromosomes (Rizzoni and Palitti 1973). This example, however, may be an exception to the rule. Moreover, the original ploidy level in *A. proteus* is not known. Taking into account all the above, clones with increased nuclear DNA content can be assumed as tetraploid and octoploid only with reserve.

Additionally, it cannot be ruled out that prolonged cultivation of the clones results in the loss of individual chromosomes as has been demonstrated for polyploid cells of mammals (Harris 1971). Due to a great number of chromosomes in *A. proteus* these losses might not be detected cytofluorimetrically.

Duration of the cell cycle of "tetraploid" and "octoploid" amoebae is not increased as compared to normal cells. This is common for the overwhelming majority of cell polyploid series (Nagl 1978, Brodsky and Uryvaeva 1981).

Still less known is how polyploidy affects cell volume. This is due to the fact that cell volumes as such are measured rather seldom. The volume is commonly estimated using indirect indices, for instance, cell area in sections (Epstein 1967), dry weight (Rigler 1962) or the amount of total protein (Sungurov 1981). A good correlation between these characters and the degree of ploidy is usually reported (Kimball 1967, Berger 1982). As regards such large cells as *A. proteus*, their volume can be determined directly (see "Materials and Methods").

According to Commandon et de Fonbrune (1942 b), the volumes of original *A. sphaeronucleus* cells and those obtained after single and double blocking of mitosis were in the ratio of 1:2:4. In our case the ratio of the volumes of original, "tetraploid" and "octo-

plaid" cells was considerably less — 1:1.2:1.7. It is still not clear whether such increase of the cell volume takes place immediately after the operation, or if the ratios show changes of cell volume occurring during prolonged cultivations in the nuclear-cytoplasmic ratio in "tetraploid" and "octoploid" amoebae in favour of the cell nucleus. A similar fact, i.e., that in polyploid rat liver cells enlarged nuclei control a relatively lesser cytoplasm volume, was reported (Žinkin et al. 1973).

Cell polyploidization is generally thought to break a volume/surface ratio (Szarski 1970, Brodsky and Uryvaeva 1981). It is likely that in the case of *A. proteus* a lesser than twofold increase of volume in "col" clones is associated with such a break. At least two hypotheses can be advanced: (1) The small increase of the cell volume in "col" clones is accounted for by the enlarged nuclear volume/surface ratio, and, as a consequence, by the decrease in the amount of RNA and/or protein synthesized (see the review by Brodsky and Uryvaeva 1981). This is supported by the fact that the cell volume increases twice in binucleate cells both in *A. sphaeronucleus* (Houssay et Prenant 1970) and, according to our preliminary data, in *A. proteus*. (2) It is not improbable that the small increase of cell volume in "col" clones is due to the increase of cell volume and cell surface ratio. While phagocytosing ciliates amoebae are known to utilize up to 10% of the plasmalemma for producing one food vacuole (Christiansen and Marshall 1965). Since with a twofold increase of cell volume the cell surface area grows only 1.6 times, it may be suggested that such amoeba would not be able to provide a sufficient amount of food for phagocytosis. It should be noted, however, that neither of the two hypotheses explains the discrepancies between our data and those of Commandon et de Fonbrune.

The viability of B2col-1 cells ("octoploids") is reduced as compared to the control. Judging by the shape of the curve showing the nuclear DNA accumulation during the cell cycle, the DNA synthesis in cells of this clone proceeds normally. In synchronized samples no dying or detached from the substratum cells were observed among those that completed their division. The decreased cloning efficiency of "octoploid" amoebae might be therefore explained by their decreased probability to complete division.

"Tetraploid" and "octoploid" clones obtained can be used in experiments on gene dosage — effect. The increased size of their nuclei may be used as a nuclear marker in producing various nuclear-cytoplasmic "hybrids".

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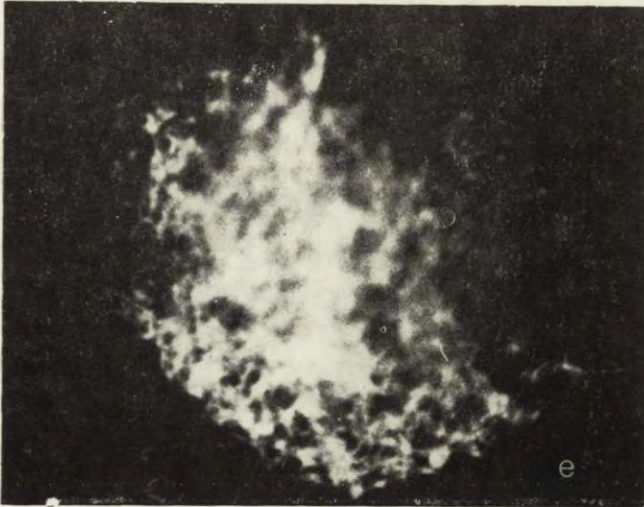
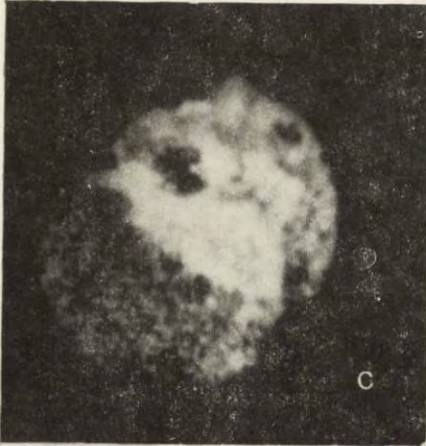
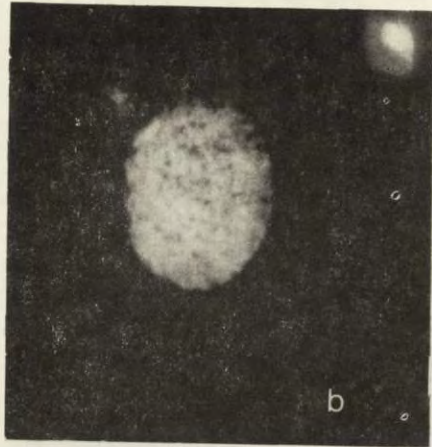
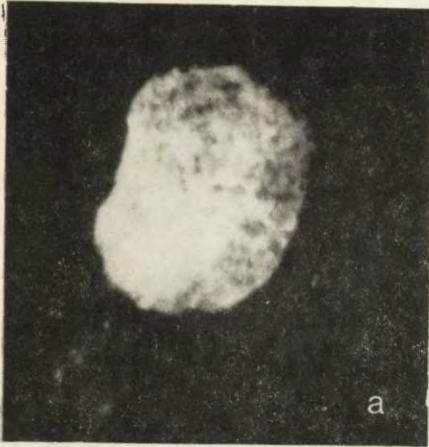
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EXPLANATION OF PLATE I

Chromosomal plates of *A. proteus* stained with Auramine 00

- a: clone Bcol-6, metaphase
- b: strain B, metaphase
- c: clone Bcol-6, early anaphase
- d: strain B, early anaphase
- e: clone B2col-1, metaphase, all at the same magnification



S. Yu. Afon'kin

auctor phot.

Trypanosoma rotatorium (Mayer, 1843) and its Experimental
Transmission through a Leech Vector, *Helobdella nociva*
Harding, 1924

Rupendu RAY and Amallesh CHOUDHURY

Zoological Survey of India, Calcutta and Department of Zoology, University of
Calcutta, 8 Lindsay Str., Calcutta-700 087, India

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Synopsis. *Trypanosoma rotatorium* is a pleomorphic haemoflagellate represented by four distinct forms viz., juvenile, slender, flat leaf-like and larger compact form, which occur in a number of heterologous anuran hosts in India. Divisional and developmental stages of *T. rotatorium* in different anuran hosts were studied. On analyzing the mensural data of *T. rotatorium* from seven host species (*Rana tigrina*, *R. limnocharis*, *R. cyanophlyctis*, *Bufo melanostictus*, *B. stomaticus*, *Rhacophorus maculatus* and *R. malabaricus*), it has been revealed that this trypanosome retained the morphometric measurements more or less within range with slight variations, possibly due to its different host interaction and ecologic variability. *T. rotatorium* was experimentally transmitted through a laboratory reared rhyncobdellid leech vector, *Helobdella nociva*. The developmental stages inside crop and gastric caeca of the leech vector have been studied and categorized as epimastigote, spheromastigote, amastigote and metacyclic forms. A possible scheme of the life-cycle of *T. rotatorium* has been suggested in the present communication.

Relatively few intermediate hosts for the trypanosomes of cold-blooded vertebrates are known. Billet (1904) was the first worker who successfully infected *Helobdella algira* with *T. rotatorium* and noted its reproduction inside the gut of the invertebrate host. Franca (1907 a, b, 1908), Nöller (1913) and Nigrelli (1944) studied the development of this parasite in *Hemiclepsis marginata*, *Placobdella parasitica* and *Macrobdeella* sp. respectively. Brumpt (1906), Barrow (1953), Buttner and Bourcart (1955) and Diamond (1958) studied the development of anuran trypanosomes in different vector leeches.

The present paper contains the detail study of the morphology and biology of *Trypanosoma rotatorium* from the Indian subregion and *Helobdella nociva*, a rhyncobdellid leech, has been experimentally demonstrated to be a possible vector for this trypanosome.

Materials and Methods

Seven species of anuran amphibians were collected from different districts of West Bengal, Assam and Nova Goa and some of them were kept alive in the laboratory. Peripheral blood was obtained from the finger tips, toes on alternate days at different intervals and was examined. Impression and spread preparations were made from liver, lungs, kidney and bone-marrow. Air-dried blood films and organ smears were fixed in 100% methanol and stained with Romanowsky type of stains and examined.

Helobdella nociva, collected from rural West Bengal were kept in finger bowls or glass vials containing aerated fresh water at room temperature. Young leeches from the cocoons hatched after 2/3 weeks. Infected *Rana tigrina* were fed by these young leeches for few minutes. After feeding the leeches were picked up gently and kept again separately in finger bowls with necessary markings.

The examination of the leeches was carried out at various set periods of time following Diamond's method (1958). For microscopical examination they were dissected after necessary narcotization in 4% aqueous Chlorobutanol (Pennak 1953). The proboscis sheath, crops, salivary glands and gastric caeca were examined. Smears were made from caecal content and crop puncture. Citrated saline solution was also used for dilution of the crop or caecal content (3:1) to study the live specimens. Air dried crop smears were fixed and stained with Romanowsky type of stains. The morphometric parameters were recorded after Hoare (1972). The photomicrographs were taken with the help of "Ergavel microscope" using PM 6 attachment camera.

Observations

(Plates I and II 1-4, 1-8)

Trypanosoma rotatorium (Mayer, 1843), a pleomorphic haemoflagellate, is (Wenyon, 1926, Nöller, 1913, Mohammed and Mansour, 1959, Bardsley and Harmsen, 1973) represented by four distinct forms viz., Type I (Juvenile), Type II (slender), Type III (flat leaf-like) and Type IV (larger compact), which occur in a number of heterologous anuran hosts (*Rana tigrina*, *R. limnocharis*, *R. cyanophlyctis*, *Bufo melanostictus*, *B. stomaticus*, *Rhacophorus maculatus* and *R. malabaricus*).

Extensive search and repeated examination through seasons revealed the presence of divisional stages of *T. rotatorium* in the peripheral

blood, liver and kidney smears, and also in the bone-marrow smears of the anuran hosts. Before longitudinal binary fission the large leaf-like trypanosome withdrew their flagella and became rounded. In *R. tigrina*, a few amastigote and epimastigote forms were detected in the blood and bone-marrow smears on the advent of winter. In *Rhacophorus maculatus*, a good number of rounded amastigote forms were detected in the liver and kidney smears and the epimastigote forms from its liver.

On analyzing the morphometric parameters of *T. rotatorium* from the above mentioned seven host species, it has been revealed that this trypanosome has retained more or less the same range of morphometric measurements with slight variations. These variations are possibly due to its different host interaction and ecologic variability.

Transmission of *T. rotatorium* from frog to leech

Trypanosoma rotatorium was experimentally transmitted through a laboratory reared Rhyncobdellid leech vector, *Helobdella nociva*. The developmental stages inside crop and gastric caeca were studied and categorized as epimastigote, spheromastigote, amastigote and metacyclic forms (Plate II 1-8). Beside these some transitional forms were also encountered. Epimastigote forms have been further subdivided into three categories (Diamond 1958) viz., long slender epimastigote, short slender epimastigote and stumpy short-membraned form, according to their shape and size of the body (Tables 1 and 2).

The noted morphological changes were involved during the transition of the vertebrate forms to invertebrate forms in the leech vector. The flagellate displayed marked slendering of the body (epimastigote and metacyclic forms), thickening of the axoneme, increased refractibility of the cytoplasm, and disappearance of the metachromatic granules and myonemes. Furthermore, the serpentine movement of the body came to a halt but the wriggling motion on its longitudinal axis was retained. The relative number of developmental forms of *T. rotatorium* in leeches on different days of infection has been enumerated in Table 2.

Transmission of *T. rotatorium* from leech to anuran hosts

The experimental transmission of *T. rotatorium* to uninfected frogs (*R. tigrina*) was accomplished by exposing the frogs to infected leeches. Subcutaneous inoculations of the proboscis contents were also made. The blood of the frogs after inoculation and leech-feeding was examined and trypanosomal forms of Type I and Type II were encountered after the 6th day of inoculation.

Table 1

Measurements of developmental stages of *T. rotatorium* in *Helobdella notica*. All measurements in μm . Mean followed by range

Flagellate forms ¹	Sample size	PK	KN	PN	NA	PA	BW	FF
Long slender epimastigotes	25	8.68 (6-14)	2.12 (2-2.5)	10.4 (8-12)	34.75 (28-42)	44.95 (36-54)	2.0 (1.8-2.5)	6.21 (5.5-7.5)
Short slender epimastigote	50	8.03 (6-10)	1.31 (1-2)	6.72 (5-9)	18.44 (13-23)	25.16 (20-31)	1.34 (1-2)	4.2 (3-5)
Stumpy short-membraned	100	5.37 (4-6)	1.21 (1-1.5)	4.43 (4-5.2)	11.77 (7-14)	16.21 (11-19)	3.14 (2.5-4.5)	2.07 (2.0-2.2)
Metacyclic	30	6.9 (4.5-8.5)	2.3 (2-3)	9.2 (8.5-10.5)	34.3 (32-38)	41.2 (40.5-42.5)	3.28 (2.5-4.2)	5.26 (3.5-7.8)

¹ Beside these forms there are amastigote and spheromastigote forms. The mean diameter of amastigote form is $7.79 \mu\text{m}$ (5.5-10) and total length of spheromastigote is $30.6 \mu\text{m}$ (29-32) width = $8.7 \mu\text{m}$ (7-10.5), free flagellum = $4.2 \mu\text{m}$ (3-5.5), nucleus = $3.6 \mu\text{m} \times 2.1 \mu\text{m}$, kinetoplast = $1.46 \mu\text{m} \times 1.04 \mu\text{m}$.

Abbreviations: PK — posterior end to the centre of the kinetoplast, KN — centre of the kinetoplast to the centre of the nucleus, PN — posterior end to the centre of the nucleus, NA — centre of the nucleus to the anterior end, PA — from the posterior end to the anterior end, BW — width of the body, FF — free flagellum.

Table 2

Relative number of developmental forms of *T. rotatorium* in leeches on different days of infection

Days after engorgement	3	4	5	7	9	11	13	16
Long slender epimastigote	105	90	0	0	0	0	0	0
Short slender epimastigote	55	70	100	110	75	25	0	0
Stumpy short-membraned	30	25	50	50	75	110	135	165
Spheromastigote	0	0	30	30	25	15	15	0
Amastigote	15	15	20	10	25	50	40	0
Metacyclic	0	0	0	0	0	0	10	35

Transitional forms are not being accounted.

Remarks

Trypanosoma rotatorium (Mayer, 1843) is highly pleomorphic haemoflagellate showing a wide host range. The present communication includes the detail study of the morphology and biology of *T. rotatorium*. Significant part of the investigation is the transmission of the haemoflagellate to laboratory reared clean leeches, *Helobdella nociva*.

A possible scheme of the life-cycle of *T. rotatorium* has been suggested in the present paper (Fig. 1) based on the study of its various developmental stages in the leech vector. Epimastigote form is the initial stage in the leech vector which passes through a sequence of changes to transitional and metacyclic forms. Division of the developmental forms are unequal like those of *T. vittatae*, *T. rajae* (Robertson 1909 a, b) and *T. pipentis* (Diamond 1958). The authors did not encounter any multiple fission of the spheromastigote stage of *T. rotatorium* in *Helobdella nociva*. The unequal division of the epimastigote in leech hosts, reported by Barrow (1953) for *T. diemyctyli* in *Batracobdella picta* and Buttner and Bourcart (1955) for *T. inopinatum* in *Helobdella algira*, corroborates well with the present study.

Three distinct types of epimastigote forms (Table 1, Pl. II 1-4, 7, 8) were encountered possibly as a result of the unequal division by longitudinal binary fission of the trypomastigote and epimastigote forms. The authors contend to designate the rounded form with short flagellum as spheromastigote, which undergoes transformation as in Diamond's (1958) Type II b and ultimately develops into stumpy short-membraned form. However, it should be reemphasized that the unequal (Diamond's Type I) development in *T. rotatorium* is by far of predominant nature. In the present investigation the authors have observed the presence of large number of metacyclic forms admixed

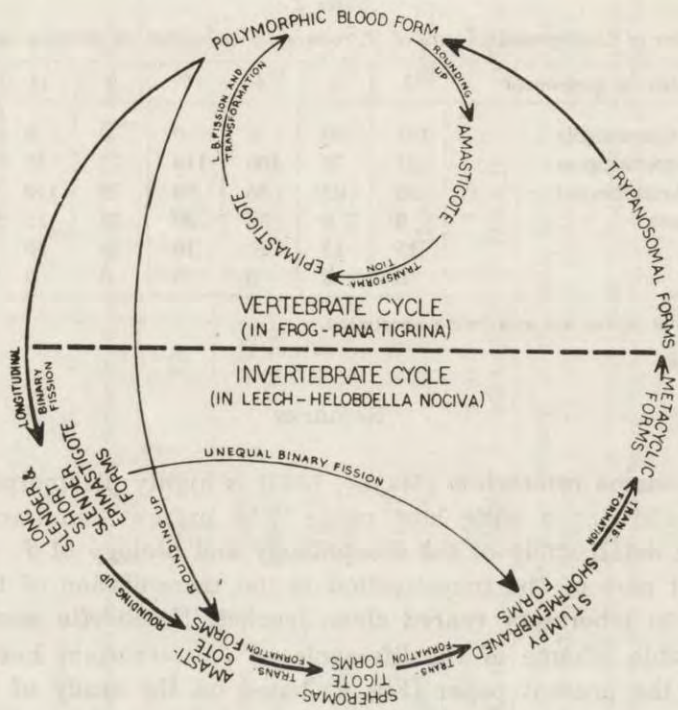


Fig. 1. Schematic representation of the life cycle of *T. rotatorium*

with a few transitional and stumpy short-membraned forms in the proboscis sheath of *Helobdella nociva*. When clean *Rana tigrina* was exposed to infected leeches, *T. rotatorium* was found to establish successfully in the anuran host.

ACKNOWLEDGEMENTS

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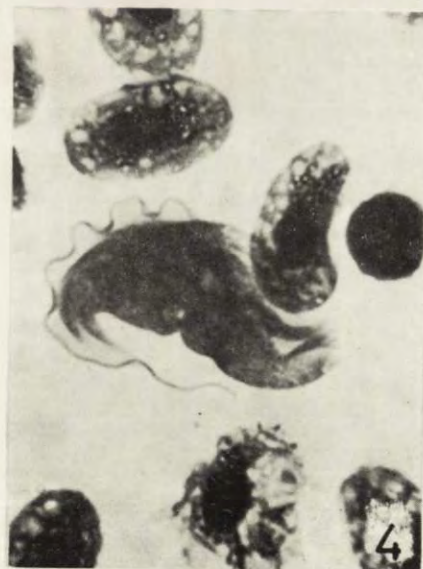
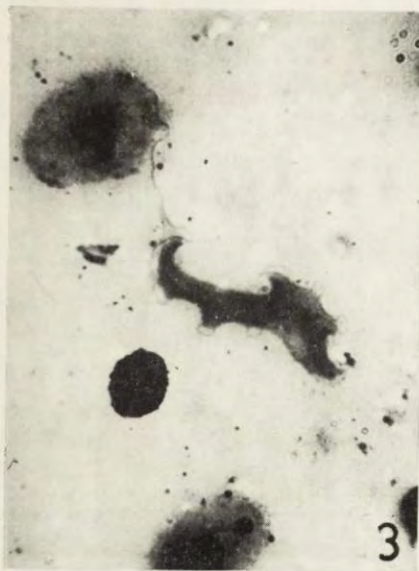
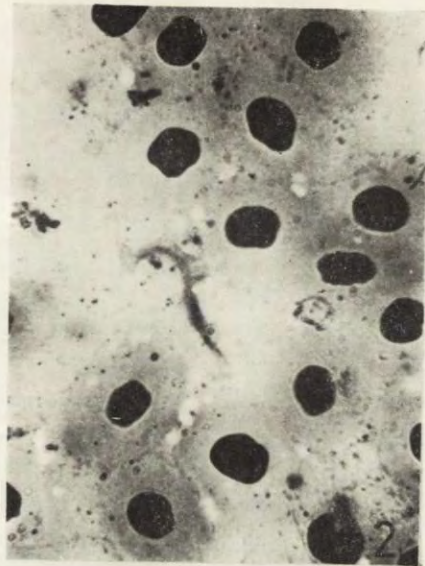
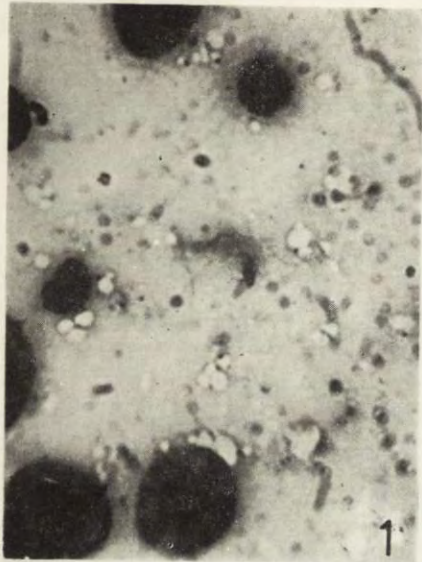
EXPLANATIONS OF PLATE I-II

Plate I. Photomicrographs of pleomorphic *Trypanosoma rotatorium* in *Rana tigrina*

- 1: Type I (Juvenile form) $\times 1500$
- 2: Type II (Slender form) $\times 1000$
- 3: Type III (Flat leaf like form) $\times 1000$
- 4: Type IV (Larger compact form) $\times 2000$

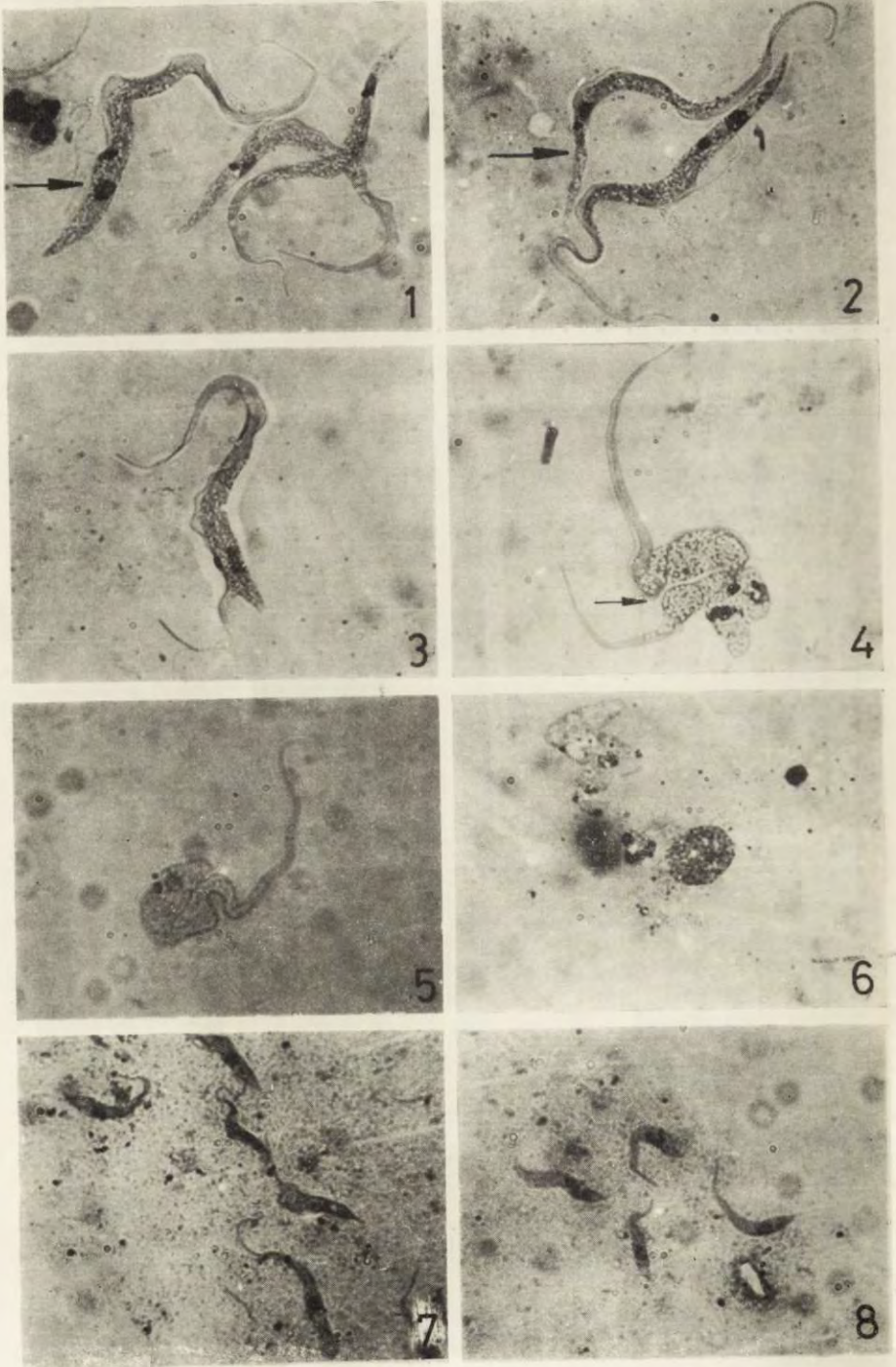
Plate II. Photomicrographs of the developmental stages of *Trypanosoma rotatorium* in leech vector, *Helobdella nociva*

- 1: Long slender epimastigote form (arrow) and two transitional forms of *T. rotatorium*, $\times 1900$
- 2: One metacyclic form (arrow) and a long slender epimastigote form $\times 1560$
- 3: A dividing (unequal L. B. fission) epimastigote form. $\times 1000$
- 4: A typical longitudinal binary fission of *T. rotatorium*, arrow indicates the line of cleavage $\times 1425$
- 5: Spheromastigote stage of *T. rotatorium*. $\times 1265$
- 6: Amastigote stage. $\times 1450$
- 7, 8: A number of stumpy short-membraned forms in the crops smear of the leech. $\times 1000$



R. Ray et A. Choudhury

auctores phot.



R. Ray et A. Choudhury

auctores phot.

A New Actinocephalid Gregarine, *Odonaticola haldari*, sp. n.,
from Odonate Insect, *Trithemis aurora* (Burmeister)

S. S. KORI and S. D. AMOJI

Department of P. G. Studies and Research in Zoology, Gulbarga
University, Gulbarga 585 106, Karnataka, India

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Synopsis. The gut contents of an odonate insect *Trithemis aurora* (Burmeister) revealed a new actinocephalid gregarine, *Odonaticola haldari*. The detailed morphology and life history of the species have been described.

During the course of studies on the cephaline gregarines of odonates from West Bengal, India, Sarkar and Haldar (1981) have created a new genus *Odonaticola* to include four species namely, *O. hexacantha*, *O. longicollara*, *O. orhethri* and *O. rodgii*. Later, Sarkar (1981) described a species, *O. elliptica* and further transferred a species *Menospora nonacontha* described by Devdhar and Deshpande (1971) to the genus *Odonaticola* on the basis of reobservations made by him. In our present investigation, we came across a new gregarine from an odonate insect, *Trithemis aurora*. Based on the morphological and various life cycle stages of the gregarine it is placed under the genus *Odonaticola* Sarkar and Haldar.

Materials and Methods

Dragonflies were collected around the Gulbarga University Campus, Gulbarga and brought to the laboratory under live condition. These were dissected under binocular microscope to take out alimentary canal in intact condition. The smears were prepared on glass slides using insect Ringer's solution as osmotic stabilizer. These were fixed in Carnoy's fluid and stained with haematoxylin. Gametocysts were collected from the hindgut region and these were placed under cavity slides along with insect Ringer's solution for providing moisture to observe the further

development. The cavity of the slide was sealed with cover glass. The heavily infected fore and midgut regions were fixed in Bouin's fluid and processed for histological observations cutting 5 μm thick sections. The dewaxed histological sections were stained as above. The illustrations given in this paper were drawn with the aid of camera lucida. All the measurements were taken from the camera lucida drawings.

Observations

The smears of the fore and midgut revealed various stages of the gregarine in its development while, those of hindgut showed the presence of gametocysts. The histological sections of the gut did not reveal the intracellular stage of the gregarine. The description of the various life cycle stages are as following:

Description of the Species

Cephalont: The three-segmented elongated cephalont (Fig. 1 A) measures about 770 μm in length and 90 μm in breadth. It has a spindle-shaped deutomerite with an elongatedly ovoidal nucleus measuring 70 μm \times 20 μm . The protomerite is dome-shaped (70 μm \times 50 μm) and at its tip originates a long slender neck of the epimerite. The neck of the epimerite is 360 μm in length and at its tip possesses a bell-shaped knob. The knob measures 25 μm in height and 25 μm in width. The periphery of the bell-shaped epimerite (Fig. 1 B) is drawn into eight petals, each with a rib-like central spine. The central region of each petal is raised on its dorsal side and gradually depresses downwards at its margin as it meets the lamina of the neighbouring petal.

Sporont: Sporonts (Fig. 1 C-E) are solitary with elongated cylindrical body ranging from 250 μm to 1750 μm in length and 75 μm to 310 μm in breadth. Since the sporadins observed are smaller than that of the cephalins, it is presumed that, the epimerite is lost at various stages of their development. The dome-shape protomerite of the early sporont changes into hemispherical-shaped as the growth proceeds. While, the deutomerite elongates enormously and assumes cylindrical in shape with conical posterior tip. The protomerite measures 140 μm \times 186 μm , and the deutomerite measures 1070 μm \times 210 μm on an average. The length of the protomerite is 4-14 times smaller than the total length (PL:TL — 1:4.16 to 13.6). The width of the deutomerite is equal to twice the width of the protomerite (PW:DW — 1:1 to 2.3). The nucleus is ovoidal or ellipsoidal measuring 71 μm \times 55 μm on an average.

Gametocyst: Freshly-formed gametocysts with partition wall (Fig. 1 F) between two gamonts are oval in shape measuring, on an average, 452 μm \times 416 μm . These are covered within a thin (50-65 μm)

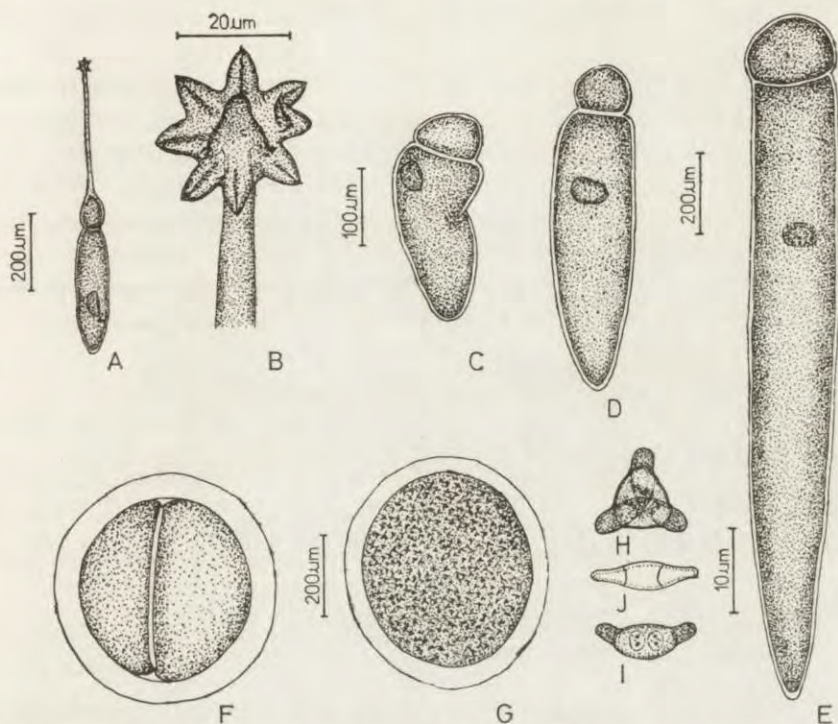


Fig. 1. *Odonaticola haldari* sp. n. A — Cephalont with elongated neck of the epimerite, B — Enlarge anterior region of the epimerite of Fig. 1 A. Note the bell-shaped knob with eight petaloid spines, C-E — Various developmental stages of sporonts, F — Freshly-formed gametocyst with partition wall and ectocyst, G — Mature gametocyst, H — Sporocysts in cluster of three. Note the triangular arrangement of the sporocysts in cluster, I — Boat-shaped sporocyst with four spherical sporozoites, J — Dorsal view of the sporocyst depicting the spindle-like structure

ectocyst. The matured gametocysts (Fig. 1 G) in moist chamber dehisce after 48 h of development by simple rupture of the cyst wall releasing the sporocysts.

Sporocyst: The sporocysts are liberated from the matured gametocysts either in clusters of three or singly. The three sporocysts in clusters are arranged in triangular (Fig. 1 H) fashion. Each sporocyst is boat-shaped (Fig. 1 I) measuring 13 μm in length and 5 μm in breadth. From the dorsal surface the sporocysts appear spindle-like (Fig. 1 J). Each sporocyst contains four spherical sporozoites.

Systematic Position

The solitary occurrence of sporadins, bell-shaped epimerite with petaloid spines at the margin, gametocysts dehiscing by simple rupture and boat-shaped sporocysts observed in the presently described gre-

garine justify its inclusion in the genus, *Odonaticola* Sarkar and Haldar.

Among the six species described so far under this genus, *O. rodgii* bears some resemblances with presently described gregarine in having eight petaloid spines in the epimerite and the size of the sporadins. However, the size and shape of the gametocysts and the size of the sporocysts of the described gregarine are markedly higher than those reported for *O. rodgii*. Hence this gregarine is considered new under the genus *Odonaticola* Sarkar and Haldar and the name *Odonaticola haldari* is proposed in honour of Dr D. P. Haldar of Kalyani University, Kalyani, India, who has contributed much to the gregarinology.

Parasite: *Odonaticola haldari*.

Host: *Trithemis aurora* (Burmeister).

Locality: Gulbarga University Campus, Gulbarga, Karnataka, India.

Site of Infection: Fore and midgut region.

Repository: The permanent slides of type specimens of this parasite are deposited in the Department of Studies and Research in Zoology, Gulbarga University, Gulbarga, Karnataka, India,

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Haemogregarina choudhuryi sp. n. (*Apicomplexa*:
Haemogregarinidae) in Common Pond Water Turtle, *Lissemys*
punctata punctata (Bonnaterre) from West Bengal

R. RAY and A. BHATTACHARJEE

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Synopsis. *Haemogregarina choudhuryi* sp. n. has been described from a common pond water turtle, *Lissemys punctata punctata* (Bonnaterre) from Bankura district, West Bengal, India. The gamonts, erythrocytic schizogony and tissue schizogony have been described in detail. The sporogonic development in the leech vector, *Helobdella nociva* Harding has also been described.

Haemogregarines were first described in 1885 by Danilewsky in a study of *Haemogregarina stepanowi* of the European tortoise *Emys orbicularis* (Linn.). Reichnow (1910) gave a detailed account of the structure and life-cycle of *H. stepanowi* in the same tortoise, as well as in the presumed invertebrate host, the leech *Placobdella cate-nigera* Muller. Hahn (1909) and later Roudabush and Coatney (1937) studied the haemogregarines in *C. serpentina* and considered it to be *H. stepanowi*. Wang and Hopkins (1965), Dessler (1973), Acholonu (1974) and Paterson and Dessler (1976) studied the life-cycle of some chelonian haemogregarines in detail.

In India, study on chelonian haemogregarine was started by Simond (1901) who described two haemogregarines, *Haemogregarina laverani* and *H. mesnili* from *Emyda granosa* and *Emys tectum* respectively. Later, Patton (1908) described *Haemogregarina nicoriae* from *Emyda granosa*. In 1912, Laveran and Nattan-Larrier described a new species *Haemogregarina testudinis* from a tortoise *Testudo emys*. Besides, three more haemogregarines viz., *H. vittatae* Robertson,

1908, *H. malabarica* de Mello, 1932 and *H. xaveri* de Mello, 1932 (Cited by Bhatia 1938) were described from a turtle *Lissemys punctata granosa* Schoepff from Nova Goa, India. Misra et al. (1974) described *Haemogregarina simondi*¹ from a river turtle *Trionyx gangeticus*. But they could neither find any erythrocytic schizogony of that haemogregarine nor the sporogony inside the leech vector.

The present communication deals with a new haemogregarine parasitizing the erythrocytes and lung endothelial cells of a common pond water turtle *Lissemys punctata punctata* along with its sporogonic development in a leech *Helobdella nociva* Harding.

Material and Methods

The turtles were collected during fishing in a pond in the village Balitha, district Bankura, West Bengal in the month of Feb.-March, 1982. Thin blood films were prepared from the blood obtained from most of the turtles by nicking the head and feet with a scalpel.

Thin blood films were then air dried, fixed in absolute methanol and stained with Romanowsky's stains. To study the tissue stages of the parasites one infected turtle was decapitated and liver, lung, kidney, heart and spleen tissues were fixed in Bouin's fixative and processed for routine histologic examination. 4-6 μm paraffin sections were cut and stained with Iron-alum haematoxylin and eosin. Organ imprints were also made from the aforementioned tissues and stained in the same manner as the blood films.

Rhyncobdellid leeches, *Helobdella nociva* Harding were found congregate in numerous numbers on the neck region, round the anal aperture and on the feet of the turtles. They were removed from the turtles and maintained in aquaria or glass vials. Five leeches were narcotized and fixed immediately after removal, 15 others were maintained in the laboratory for several weeks and then allowed to refeed. Five unfed leeches were fixed as controls.

Concurrent with the fixation of whole leeches, smears of the contents of the gastric and intestinal caeca of other leeches were prepared.

The narcotized leeches were stretched by pinning them with insect pins through the anterior and posterior suckers onto cardboard strips. The ends of the pins were trimmed and the strip introduced into vials containing Bouin's fixative and processed for routine histologic examinations. The leeches were sectioned serially (4-6 μm) and stained with Iron-alum haematoxylin and eosin.

Camera-lucida drawings of the parasites were made and photomicrographs were taken with the help of an Olympus PM6 attachment camera.

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

¹ Since the name *H. simondi* was preoccupied, Misra (1976) emended it to *H. gangetica*.

Observations

Haemogregarina choudhuryi sp. n.
(Pl. I 1-12 and Figs. 1 a-h, 2 a-g)Type-host: *Lissemys punctata punctata* (Bonnaterre)

Type-locality: Balitha, Bankura, West Bengal

Site of infection: Erythrocytes and lung tissue

Prevalence: Of 5 turtles examined, 3 (60%) were found to be infected

Vector: *Helobdella nociva* Harding

Description

Young gamonts (Pl. I 1-12 and Fig. 1 a)

These are elongated or "comma shaped" with one end narrower than the other, measure 5.0 by 1.5 μm with an average area of 8.0 μm^2 ($N = 10$). The cytoplasm is densely granulated and stained light blue with Leishman stain. The central oval nucleus measures 1.5×0.7 μm

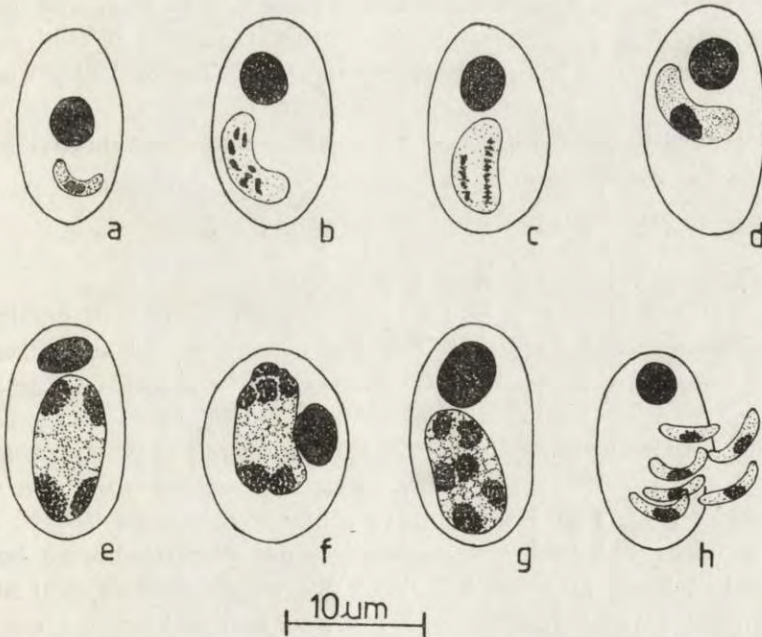


Fig. 1 a-h — *Haemogregarina choudhuryi* sp. nov. in the blood of pond water turtle *Lissemys punctata punctata* (Bonnaterre). a — young gamont, b-c — microgamonts, d — macrogamont, e-g — erythrocytic schizogony, h — the merozoites releasing from the erythrocyte

which stained pink colour with Leishman. They are situated either beside the nucleus or at one end of the erythrocytes. In this stage the sexes cannot be differentiated.

Mature Gamonts (Pl. I 2-5)

They are bean-shaped with both the ends bluntly rounded, measure $8.0 \times 2.5 \mu\text{m}$ with an average area of $18.7 \mu\text{m}^2$ ($N = 10$). The cytoplasm stained blue with Leishman and some metachromatic granules were also found evenly distributed throughout the cytoplasm. They are situated either on one half of the erythrocyte facing their concave border towards the convex side of the host cell nucleus or in any position in the host cell. The sex of the mature gamonts is apparent. The microgamonts are 'kidney-bean' shaped with a few metachromatic granules and stains light blue with Leishman. The nucleus is not at all compact but either band-shaped adhering to the opposite side of the cell membrane along the longitudinal axis of the parasite or may be in the form of 6-10 chromatin granules. They measure $8.5 \times 2.0 \mu\text{m}$ with an average area of $15.5 \mu\text{m}^2$ ($N = 10$). The macrogamonts are elongated, slightly curved with both the ends equally blunt. The cytoplasm stains deep blue with Leishman and a good number of red stained metachromatic granules were found uniformly distributed. The central nucleus is oval or rounded and stains deep red with Leishman and Giemsa. They measure $8.5 \times 3.5 \mu\text{m}$ with an average area of $20.6 \mu\text{m}^2$ ($N = 10$).

As the parasite enlarges the cytoplasm became vacuolated and the division of the nucleus starts.

Schizogony (Pl. I 6-12)

Two types of schizogony were recorded in the turtles.

Erythrocytic Schizogony: A good number of erythrocytic schizonts were noted in the red blood corpuscles of the circulating blood. As the parasites mature the nuclear division ensues. Binucleate, tetranucleate and hexanucleated schizonts are of very common occurrence. Successive divisions culminate in the formation of six merozoites. Before release, the merozoites may be seen arranged around a central residual mass (Fig. 1 g). Each mature schizont measures $10.0 \times 5.5 \mu\text{m}$ with an area of $38.5 \mu\text{m}^2$. The merozoites are elongated with both the ends tapering. They measure $5.7 \times 1.0 \mu\text{m}$ with a central oval nucleus.

Tissue Schizogony (Pl. I 10-12): Tissue schizogony was noted only in the endothelial cells of lung capillaries. Other organs viz., liver, spleen, kidney etc., remain negative. Each mature schizont is oval in structure measuring $16.5 \times 10.0 \mu\text{m}$ which contains 20-35 developing merozoites around a central residual mass. Each merozoite measures $4.0 \times 1.2 \mu\text{m}$.

Effect of the Parasite on the Host Cell

The intraerythrocytic parasite has got some direct effect on the host cell and its nucleus. The parasitized erythrocyte became hypertrophied with displacement of its nucleus. Sometimes the host cell nucleus pushed at the pole or totally ejected in case of double infection.

Development Inside Leech-vector (Fig. 2 a-g)

In the gastric caeca of the leech *Helobdella nociva*, the blood stages are released from the turtle erythrocytes. The gametocytes remain unchanged for sometimes. In the intestinal caeca micro- and macrogamonts are found on the microvillar surface of the epithelial cells. A single macrogametocyte gives rise to a single macrogamete. While a single microgametocyte gives rise to four nonmotile microgametes by two successive divisions. Oocyst develops by the union of a macrogamete and a microgamete on the endothelial surface. Young oocyst measures $6.5 \times 7.0 \mu\text{m}$. The mature oocyst measures $15.5 \times 12.5 \mu\text{m}$ which contains 8-10 sporozoites. The sporozoites are elongated measuring $10.5 \times 2.5 \mu\text{m}$ (N = 5). The nucleus, located in the midregion of

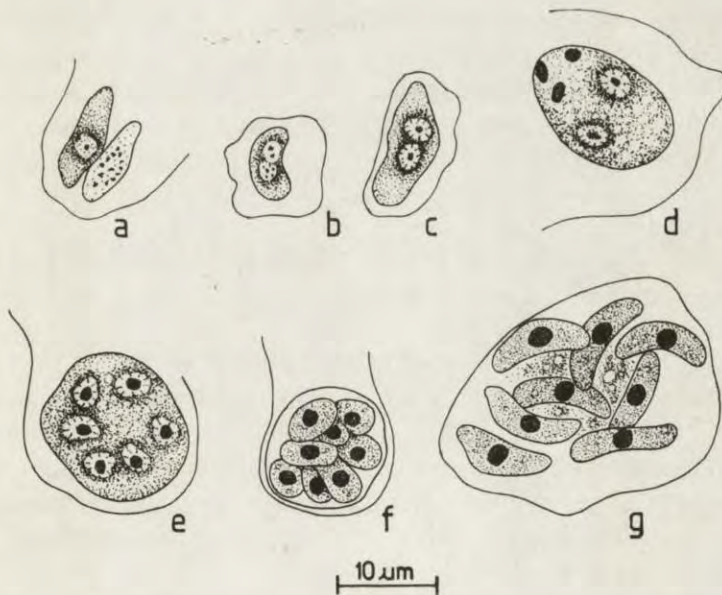


Fig. 2 a-g — Different stages of sporogonic development of *Haemogregarina choudhuryi* sp. nov. in the intestinal caeca of leech vector, *Helobdella nociva* Harding. a — gametic association on the microvillar surface, b-e — different developmental stages of early oocyst, f — a mature oocyst containing eight immature sporozoites, g — eight fully developed sporozoites released in a large parasitophorous vacuole

the body, is roughly ovoid in shape measuring $2.5 \times 2.0 \mu\text{m}$. Sporozoites liberated from the oocyst are found in the tissues of the leech.

Diagnosis of *Haemogregarina choudhuryi* sp. n.

The macro- and microgamonts of *H. choudhuryi* are "kidney-bean" shaped measuring $8.5 \times 3.5 \mu\text{m}$ with an average of area of $20.6 \mu\text{m}^2$ and $8.5 \times 2.0 \mu\text{m}$ with an average area of $15.5 \mu\text{m}^2$ respectively. Immature erythrocytic schizonts are binucleated, tetranucleated and hexanucleated. The mature erythrocytic schizont measures $10.0 \times 5.5 \mu\text{m}$ with an area of $38.5 \mu\text{m}^2$ which produces six merozoites. The merozoites measure $5.7 \times 1.0 \mu\text{m}$. The tissue schizonts are found only in the endothelial cells of lung capillaries. Each mature schizont is oval measuring $16.5 \times 10 \mu\text{m}$ which contains 20–35 merozoites.

Discussion

A review of the literature reveals that there are several different species of *Haemogregarina* in chelonians.

Haemogregarina choudhuryi has got some resemblances with *H. balli* and *H. stepanowi* by having similar type of erythrocytic schizonts with 6–8 merozoites and the sporogonic stages in the leech appear similar. But it differs from them in the type and localization of schizonts. Reichnow (1910) recorded schizonts with 12–24 merozoites in erythrocytes, in the bone-marrow and Patterson and Dessler (1976) recorded the schizont of *H. balli* in a variety of cells in the liver, lung and spleen. In contrast *H. choudhuryi* has schizonts only in the endothelial cells of lung capillaries.

The present species differs from *H. nicoriae* in structure and location of the tissue schizonts and the clear differentiation of the sex of the gamonts. *Haemogregarina pseudemydis* lacks tissue schizonts and does not have erythrocytic schizonts with 6–8 merozoites. Acholonu (1974) observed 35–140 merozoites in a schizont in the erythrocytes and leucocytes which is unlike any previously described species of *Haemogregarina*. In *H. pseudemydis* the sporogonic development is also unknown.

The parasite under report differs from all the previous haemogregarines so far described from tortoises and turtles in morphometric measurements and other characteristics in details.

So it has been assigned a new name *Haemogregarina choudhuryi* sp. n. after the name of Dr. Amalesh Choudhury, a renowned protozoologist of India.

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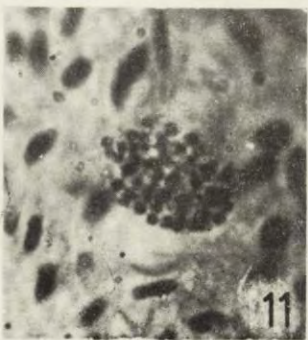
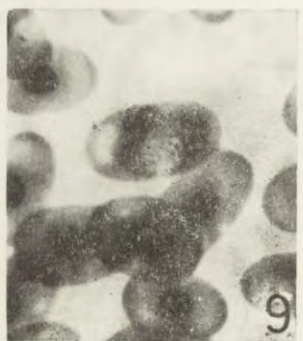
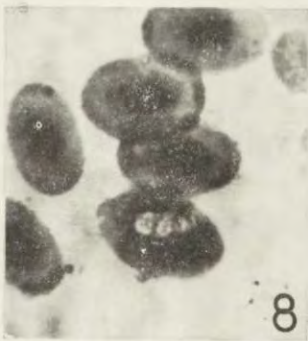
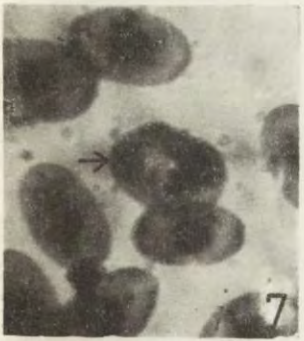
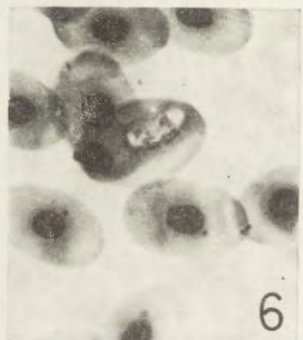
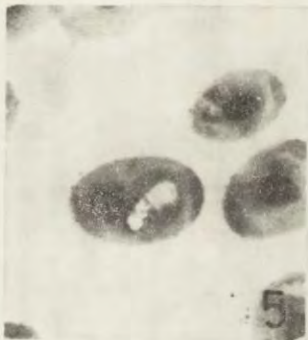
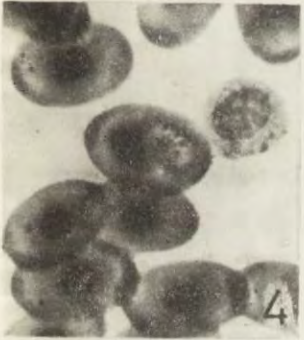
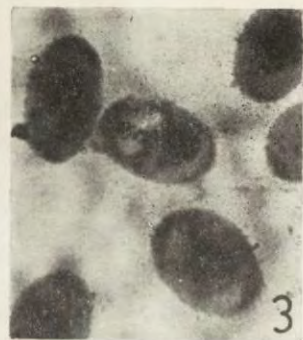
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Addendum: Since the manuscript was sent for publication, we have come across a paper in which Misra K. K., 1981 (*Proc. Zool. Soc., Calcutta*, 32: 141-143) described the erythrocytic schizogony of *Haemogregarina gangetica* from a river turtle *Trionyx gangeticus*, which is quite different from *H. choudhuryi* sp. n. described in the present paper.

EXPLANATION OF PLATE I

- 1: Gametocytes in the erythrocytes of *Lissemys punctata punctata* × 1050
- 2-3: Female or Macrogametocytes × 1050
- 4-5 Male or Microgametocytes × 1050
- 6: An early binucleate erythrocytic schizont × 1050
- 7: A dividing schizont showing the accumulation of nuclear materials at the poles of the parasite × 1050
- 8-9: Mature erythrocytic schizonts with 6 developing merozoites × 1050
- 10: An early schizont in the endothelial cells of lung tissue of the host × 1212
- 11: A multinucleated schizont in the same tissue of the turtle × 1210
- 12: A mature schizont with developing merozoites × 1210



R. Ray et A. Bhattacharjee

auctores phot.

In preparation:

C. Groliere et M. Couteaux: Morphologie et infracillature de *Kahlilembus fusiformis* (Kahl 1926) gen. nov., scuticocilie du sol — E. Mikołajczyk: The step-up Photophobic Response in *Euglena*: 2. Sensitivity to Light of the Colorless Flagellate *Astasia longa* in Low and High Viscosity Medium — J. Sikora and A. Jurand: Possible Role of Cilia in the Control of Cytoplasmic Streaming in the Control of Cytoplasmic Streaming in *Paramecium aurelia* — A. Wasik and J. Sikora: Effect of External Agents on Cytoplasmic Streaming in *Paramecium*. II. Influence of Media Free of Suspension — A. Wasik and J. Sikora: Effect of External Agents on Cytoplasmic Streaming in *Paramecium*. III. Influence of Endocytosis Cessation — A. Grębecki and M. Cieślawska: Motive Force Generation Site in Plasmodium of *Physarum polycephalum*, a Dissection Study — C. Kalavati and C. C. Narasimhamurti: Two New Species of Myxosporidians, *Chloromyxum mitchelli* sp. n. from Fish *Therapon jarbua* (Forsskal)

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Bieżące i archiwalne numery można nabyć lub zamówić we Wzorcowni Ośrodka Rozpowszechniania Wydawnictw Naukowych PAN, Pałac Kultury i Nauki, 00-901 Warszawa oraz w księgarniach naukowych „Domu Książki”.

CONTENTS

E. Mikołajczyk: Photophobic Responses in <i>Euglenina</i> . 1. Effects of Excitation Wavelength and External Medium on the Step-up Response of Light- and Dark-grown <i>Euglena gracilis</i>	1
M. Kiersnowska: Studies on Cell Adaptation: Effects of a Cycloheximide on <i>Euplotes minuta</i>	11
M. Kiersnowska and L. Jasińska: Effects of Continuous Presence of Actinomycin D on the Clonal Growth of <i>Euplotes minuta</i>	27
S. Yu. Afon'kin: Production of <i>Amoeba proteus</i> Clones with Increased Nuclear DNA Content	35
R. Ray and A. Choudhury: <i>Trypanosoma rotatorium</i> (Mayer, 1843) and its Experimental Transmission through a Leech Vector, <i>Helobdella nociva</i> Harding, 1924	55
S. S. Kori and S. D. A moji: A New Actinocephalid Gregarine, <i>Odonaticola haldari</i> , sp. n., from Odonate Insect, <i>Trithemis aurora</i> (Burmeister)	63
R. Ray and A. Bhattacharjee: <i>Haemogregarina choudhuryi</i> sp. n. (<i>Apicomplexa</i> : <i>Haemogregarinidae</i>) in Common Pond Water Turtle, <i>Lissemys punctata punctata</i> (Bonnaterre) from West Bengal	67
VII International Congress of Protozoology: 22-29 June 1985, Nairobi, Kenya Organizing Committee: P.O. Box 60444, Nairobi, Kenya Chairman: Prof. Thomas R. ODHAMBIO; Secretary: Dr. L. H. OTIENO	

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Index 35133