ACTA PROTOZOO-LOGICA

Die Ausbildung von drei Membranellen im Verlauf der Stomatogenese von *Drepanomonas revoluta* Penard (*Ciliata, Microthoracina*)[1]

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Synopsis. Die Stomatogenese von *Drepanomonas revoluta* wurde anhand von Protargol und Silbernitrat gefärbten Tieren untersucht. Sie ist telokinetal. Stomatogen ist eine Somakinete. An ihr proliferieren Kinetosomen und bilden ein Primordialfeld. Der aus drei Membranellen aufgebaute Oralapparat der *Microthoracina* ist nur während der Teilung sichtbar.

Drepanomonas-Arten sind typische Bewohner im Kapillarwasser von Moosen Kahl (1930–35), Wenzel (1953). Nach Buitkamp (1977) und Dragesco et Dragesco-Kerneis (1986) lebt *Drepanomonas revoluta* außerdem auch im Boden. Wir fanden diese Art in einem anderen kapillaren Lebensraum, nämlich im Mesopsammal des Humboldt See, Saskatchewan, Kanada. Die Infraciliatur von *D. revoluta* ist im Prinzip bekannt. Wir haben uns deshalb auf die Untersuchung der bislang unbekannten Morphogenese des Oralapparates bei der Zweiteilung beschränkt.

Material und Methode

Die Sandlückenfauna wurde nach der Methode von Uhlig (1964) mit Eis ausgetrieben, das in diesem Falle aus Wasser des Humboldt Sees hergestellt worden war. Aus der so gewonnenen Probe ist *D. revoluta* isoliert und dann in Petrischalen kultiviert worden. Die Tiere wurden mit Bakterien gefüttert, die sich an einem in die Kultur gebrachtes Reiskorn entwickelten. Die Infraciliatur der Tiere wurde mit Silbernitrat nach Chatton-Lwoff (1930) und Protargol nach Wilbert (1975) untersucht. Zur Charakterisierung des Biotops, aus der unsere Population stammt, ist zu sagen, daß der Humboldt See ein Magnesiumsulfat-See ist mit einer Salinität von etwa S = 4. Zur Limnologie dieses und anderer saliner Seen in Saskatchewan s. Hammer (1978).
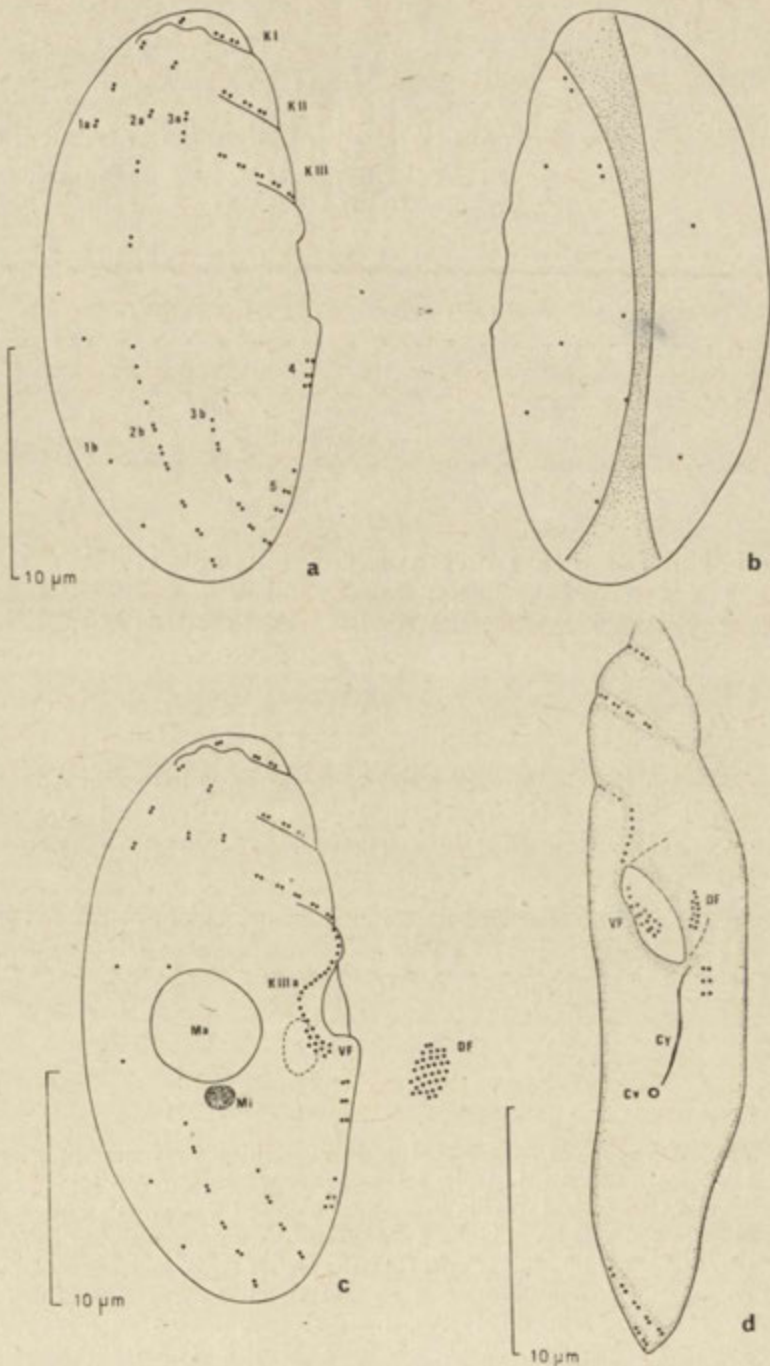
Abb. 1. *Drepanomonas revoluta* Penard, a — ventrales und b — dorsales Kinetom. c — Orale Infraciliatur. Imprägnation mit Protargol. d — Ansicht der Ventralkante. Versilberung nach Chatton-Lwoff. Cv — Exkretionsporus, Cy — Cytopyge, DF — Dorsalfeld, VF — Ventralfeld, Ma — Makronucleus, Mi — Mikronucleus
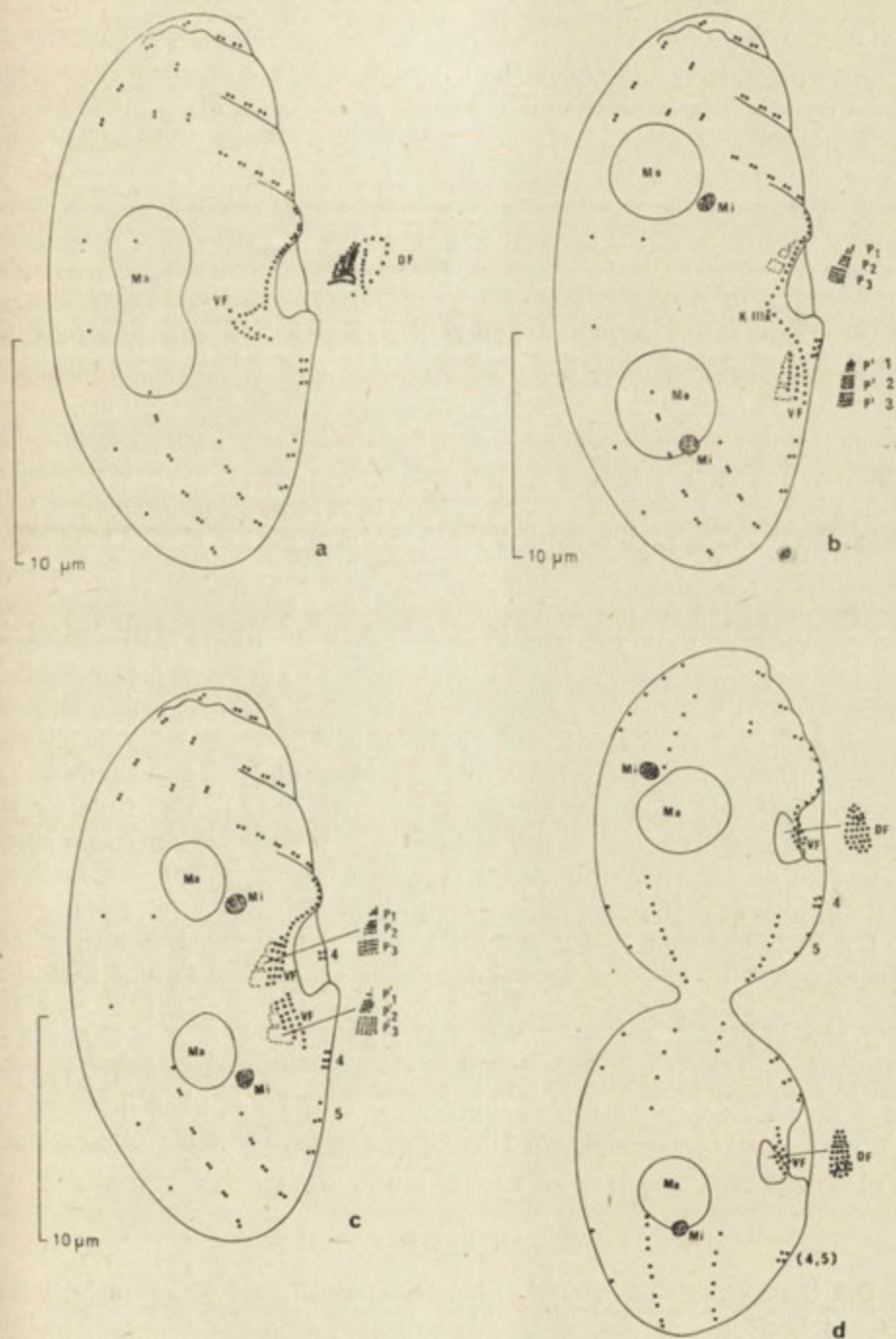
Abb. 2. *Drepanomonas revoluta* Penard. Stomatogenesestadien nach Imprägnation mit Protargol. DF — Dorsalfeld, Ma — Makronucleus, Mi — Mikronucleus, P, P' — Membranellen, VF — Ventralfeld

Ergebnisse

Drepanomonas revoluta (Abb. 1a–d), Gestalt bohnenförmig, um 34 μm lang, 13 μm breit. Mundgrube median in der linken Körperkante. Ein zentraler Makronucleus (Ma), benachbart ein Mikronucleus (Mi), Cytopyge (Cy) und Pore der kontraktilen Vakuole (Cv) postoral auf der Ventralkante.

Die Population von Buitkamp aus den Böden der „savane brulée" Rep. Elfenbeinküste, Afrika, hat ein kurzes Kinetenfragment ventral auf der Außenkante der Mundgrube. Es fehlt unseren Tieren. Der Hauptunterschied beider Populationen zeigt sich aber im Oralapparat. Buitkamp findet drei kleine in einer Ebene stehende Membranellen. Wir fanden in der Mundgrube zwei getrennte Felder, nämlich ein ventrales Feld (VF) aus drei Kineten und diesem dorsal gegenüber ein größeres Feld, das Dorsalfeld (DF). Wie sich später zeigen wird, nimmt die Kinete III der Bewimperung eine Sonderstellung ein. Im Anfang besteht sie aus gepaarten Kinetosomen. Dann verlängert sie sich zu einer Reihe einzelner Kinetosomen, die bis in die Mundhöhle herein zum Ventralfeld reichen. Dieser Teil hat hier die Bezeichnung K III a.

Die Stomatogenese ist anfänglich an einer Auflösung der Kinetosomenanordnung im Dorsal- und Ventralfeld zu erkennen. Diese Umorientierung ist von einer starken Ausweitung der Mundbucht in Längsrichtung begleitet. Als nächstes wandert das Ventralfeld zum kaudalen Ende der Bucht. Die Kinete K IIIa verlängert sich bleibt aber weiter mit dem Ventralfeld verbunden. Schon in diesem Stadium haben sich zwei Dorsalfelder ausgebildet, die in auffälliger Weise in je drei Membranellen P 1, 2, 3 in der vorderen und P′ 1, 2, 3 in der hinteren Zellhälfte differenziert sind. Der Kernapparat ist geteilt (Abb. 2 a, b). Im nächsten Stadium (Abb. 2 c) hat das Ventralfeld durch eine Proliferation von Kinetosomen vom Ende der Kinete K IIIa an Größe zugenommen und teilt sich. Die Dorsalfelder zeigen immer noch einen dreiteiligen Aufbau. Späte Teilungsstadien (Abb. 2 d) schließlich zeigen im Proter und Opisthen wieder kompakte Dorsal- und Ventralfelder.

Im Proter bleibt die Verbindung K III–K III a immer bestehen. Im Opisthen wird sie später neu hergestellt, wobei den Kinetosomenfeldern 4 und 5 (Abb. 1 a) eine besondere Bedeutung spielen. Feld 4 des Elterntieres teilt sich (Abb. 2 c). Die vorderen Kinetosomen differenzieren sich später zu Feld 4 und 5 des Proter (Abb. 2 d). Im Opisthen schiebt sich die neue Mundbucht zwischen die Felder 4 und 5. Von den Kinetosomen des vorderen Feldes, also dem ursprünglich 4., leiten sich dann die Kineten I, II, III ab und vom Feld 5 des Elterntieres schließlich die Felder 4 und 5 des Opisthen.

Diskussion

Der Oralapparat von *D. revoluta* hat im Vergleich zu den Genera Microthorax und Leptopharynx einen untypischen Aufbau, denn hier sind nur ein Dorsales- und Ventrales Wimperfeld vorhanden, während jene 3 Membranellen und eine periorale Kinete besitzen. Die Teilungsstadien zeigen aber, daß das Dorsalfeld in Wirklichkeit aus drei Membranellen besteht, indem es zunächst in drei Mem-

branellen zerfällt und später aus drei Membranellen neu aufgebaut wird. Auch die periorale Kinete ist nur während der Teilung sichtbar, als solche betrachten wir nämlich den Kinetosomenschweif, der bei der Umwandlung des Dorsalfeldes (Abb. 2 a) entsteht.

D. revoluta, Leptopharynx Njiné (1978), Microthorax Peck (1975) haben eine telokinetale Stomatogenese. Hier ist die stomatogene Kinete die K III a, denn an ihrem Ende proliferieren Kinetosomen. Sie formieren sich zu dem hier so bezeichneten Ventralfeld, das nach Lage und Herkunft aber ein Primordialfeld ist. Aus ihm differenzieren Leptopharynx und Microthorax den neuen Oralapparat. Obwohl wir die weitere Entwicklung des Primordialfeldes nicht verfolgen konnten, ist wegen der grundsätzlichen Übereinstimmung in der Stomatogenese und im Aufbau des Oralapparates mit den beiden anderen Genera davon auszugehen, daß sich auch hier die Membranellen vom von diesem Feld herleiten.

Nach Buitkamp (1977) hat *D. revoluta* einen Oralapparat aus 3 membranellenartigen Gebilden. Da seiner Population die Kinete K III a fehlt, ist zu vermuten, daß sie als Fragment in der Mundgrube existiert. Dieses zusammen mit dem Promordialfeld und dem Verschmelzungsprodukt der drei Membranellen, zeigen dann den dreiteiligen Aufbau. Diese Annahme ist aber letztlich nur durch die Untersuchung der Stomatogenese dieser Population zu klären.

SUMMARY

The stomatogenesis of *Drepanomonas revoluta* was studied by means of protargol and silver nitrate methods. The stomatogenesis is telokinetal. During division one somatic kinety extending from ventral into the buccal cavity proliferates kinetosomes forming the later primordial field. Only during stomatogenesis *Drepanomonas revoluta* exhibits one special feature of *Microthoracina*: the three membranelles of the buccal apparatus.

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Distribution of *Dileptus anser* (*Ciliata, Holotricha, Gymnostomatida*) of Various Genotypes for *mat* Locus in the Concentration Gradient of Their Gamones

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Synopsis. When placed into the agar-chamber depression with concentration gradient of a heterologous (i.e., released by cells of a complementary mating type) gamone, ciliates accumulate within the zone of its highest concentration. The response was used to assay the kind of gamones excreted by ciliates of various mating types (MTs). It was shown preliminarily that two different gamones mixed even in the ratio 16:1 might be detected in the mixture by means of the bioassay. Ciliates of each of the three MTs, when homozygous in *mat* locus, release a specific kind of gamone(s). As to the *mat* heterozygotes, their gamone induces aggregation of only one of the two corresponding homozygotes. It is assumed, therefore, that a heterozygote excretes only one kind of gamone(s) similar to that of a homozygote with the same MT.

Clones of ciliates are usually classed as different mating types (MTs) when homological and/or heterological pairs of cells are formed in their mixture, i.e., the sexual process begins in cells of at least one clone but no pairs arise in non-mixed cultures. The genetics of MTs has been studied in a sufficiently great number of ciliate species (Sonneborn 1977). Besides it is known that some ciliates release to the culture medium specific signal substances, gamones, that trigger initial stages of conjugation. It seems likely that certain relationships exist between the gamones and the formal genetics of MTs.

For American strains of *Euplotes patella* (Kimball 1942) and another syngen of the species (Akada 1985) and also for *E. octocarinatus* (Heckmann and Kuhlmann 1982, 1986) bioassays were carried out to estimate the excretion of gamones and their qualitative composition, namely, observations were made over effects exerted by cell-free culture fluids (CFFs) on the clones under analysis. The keypoint of these observations in that CFF of the heterozygous clone $mat^x mat^y$ induced selfing in clones of a $mat^x mat^x$ genotype. The reciprocal combination pro-

duced no such effect (see, however, Luporini and Miceli 1984). These facts are generally interpreted as follows: the CFFs of clones heterozygous in *mat* locus contain a gamone that is lacking in CFFs of the corresponding homozygous clones. By contrast, the CFFs of the both homozygous clones contain no gamones that would act as heterologous to the corresponding heterozygous clones. A conclusion is commonly drawn that while the homozygous clones $mat^x mat^x$ or $mat^y mat^y$ produce only one gamone (X or Y), the heterozygotes $mat^x mat^y$ excrete a mixture of X and Y gamones. Indeed, in *E. raikovi* two species of gamones released to the medium by one of the clones heterozygous in *mat* locus were separated using gel filtration (Miceli et al. 1985). It must be emphasized that in all the above *Euplotes* species alleles of *mat* locus are co-dominant (Kimball 1942, Heckmann and Kuhlmann 1982, 1986, Miceli et al. 1983, Luporini and Miceli 1984, Akada 1985).

The results were different when we analysed the genetic determination of MTs and the ability of clones of a known genotype in *mat* locus to produce gamones in another ciliate, *Dileptus anser.*

Material and Methods

Clones of *D. anser* isolated from a natural population near Leningrad in autumn of 1984 and clones obtained in result of the crosses between them were used for the experiment (Table 1). The clones were maintained in a collection of protozoan cultures of the Laboratory (Institute of Cytology of the Academy of Science of the USSR) according to Nikolaeva (1968), i.e., cells were cultured in the salt medium (Prescott and Carrier 1964) at 25°C using *Tetrahymena pyriformis* as food.

Two complementary clones were crossed through placing cells in pairs into the wells of microplates (Afon'kin and Yudin 1985). Cells derived from conjugating pairs after conjugation divisions (Tavrovskaya 1974) were removed from the wells. Clones were grown from exconjugant cells, and after a period of immaturity, which in our experiments lasted for 3 or 4 months (provided 1–2 divisions a day) the MT was assayed.

To do this, 5–10 cells from each of the three standard clones of MTs I, II and III were put into the wells. Thereupon 5–8 cells of the clone under analysis were added to each well. One or two hours later conjugating pairs were formed in wells containing cells complementary in MT. Thus we would unambiguously class the clone under test with one of the three MTs. To control the mating reactivity of standard clones conjugation was induced each time by mixing three standard clones by two.

Selfing was induced by transferring cells to a complementary CFF (Tavrovskaya 1974).

The experiments were performed in agar chambers (technics frequently employed for studying chemotaxis in protozoans, for instance by Nohme and Tawada 1976). Forty mm plastic Petri dishes were filled with 2% agar (Difco) prepared on the Prescott-Carrier medium. Two cylindric wells with the diameter 5.8 mm and the depth of about 2.7 mm (down to the dish bottom) were cut in thickened agar 1 mm apart (Fig. 1).

Into one of the wells were placed 50 cells of a clone under test with CFF from the same culture, and into the other — either 50 cells of the same clone (Control 1) or CFF of the latter (Control 2) or 50 cells of a complementary clone (Experiment 1) or, finally, CFF of the latter (Experiment 2).

The content of one well could diffuse into the other across a thin agar isthmus between them, and, partially, beneath it. A concentration gradient of diffusing substances was supposed to arise in the neighbouring depression, oriented along the axis connecting the well centres. The highest concentration zone was right near the narrowest portion of the isthmus.

Cells were taken for the experiment from mass cultures a day after the last feeding. To obtain CFF, 100–150 ciliates were cultured in plastic microaquaria (0.2 ml in volume) during 24 h. Thereupon a medium devoid of cells was picked up using a micropipette.

The degree of unevenness in the distribution of ciliates in the well was estimated. To this purpose the wells were photographed over definitie intervals with the aid of a camera attachment MP-5.

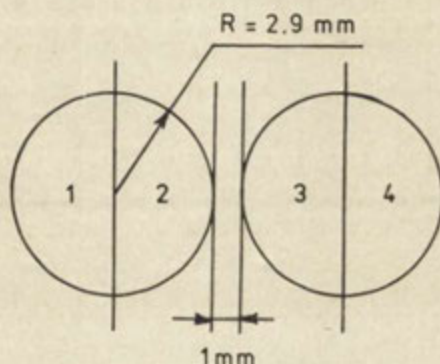In the negatives the wells were divided into the right and left halves (Fig. 1) and the number

Fig. 1. Schematic representation of parabiotic agar chamber

of cells was counted in each. Then the difference between cell numbers in two halves of the well was determined and expressed in percent of the total cell number. When cells were more numerous in a half proximal to the neighbouring well (2 and 3 in Fig. 1) the difference was denoted by "+" and in the opposite case (1 and 4 in Fig. 1) by "−" (cf. Honda and Miyake 1975). Random fluctuations of the difference may attain maximally 24% (Chi-Square at $\alpha = 0.05$). A greater difference is an indication of uneven distribution of cells between the right and left halves of the well.

Results

Genetic experiments described in detail elsewhere (Yudin and Afon'kin 1987) favour the hypothesis that clones Nos. 11, 20 and 12 of MT I, II and III, respectively, are homozygous in *mat* locus (Table 1). Using the agar well method we managed to show that (1) all the three clones excrete gamones to the medium, and (2) spatial distribution of cells in the gamone gradients is dramatically asymmetric.

If ciliates of clones under test (Nos. 11, 20 or 12, Control 1, Fig. 2, curves 3 and 4) are put vs. cells of the same clones, unevenness in the cell distribution between the right and left halves of the both wells never exceeds the level of random fluctuations within 2 h from the onset of the experiment. The picture was different when cells of complementary MT were placed into the neighbouring well (Experiment 1,

Table 1

Clones of *D. anser* used in the experiment

Mating type	Clone No.	Origin	Genotype in *mat* locus
I	11	From nature	$mat^1 mat^1$
	34	$F_1(11 \times 11)$	$mar^1 mat^1$
	35	$F_1(11 \times 11)$	$mat^1 mat^1$
	49	$F_1(11 \times 20)$	$mat^1 mat^2$
	50	$F_1(11 \times 20)$	$mat^1 mat^2$
II	20	From nature	$mat^2 mat^2$
	40	$F_1(20 \times 20)$	$mat^2 mat^2$
	56	$F_1(20 \times 8)$	$mat^2 mat^3$
	57	$F_1(20 \times 8)$	$mat^2 mat^3$
	61	$F_1(20 \times 8)$	$mat^2 mat^3$
	65	$F_1(20 \times 12)$	$mat^2 mat^3$
III	8	From nature	$mat^3 mat^3$
	12	From nature	$mat^3 mat^3$
	28	From nature	$mat^3 mat^3$

Fig. 2, Curves 1 and 2). Half an hour or one hour after the onset of the experiment a greater portion of cells in the both wells was found to aggregate in a half proximal to the neighbouring well. Soon after all (or nearly all) cells in two wells accumulated on the both sides of the thinnest part of the agar isthmus (Fig. 3) and stayed there throughout the experiment (6 h).

Such pattern is common to all of the three possible pair combinations of complementary clones Nos. 11, 20 and 12. A more detailed description of the experiments has been given previously (Afon'kin and Yudin 1986).
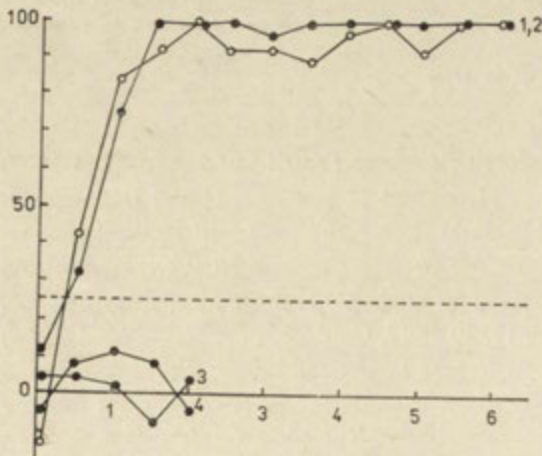
Fig. 2. Experiment 1. An example of distribution of complementary type cells in parabiotic chambers. The open circles mark clone No. 11, the black circles, clone No. 12. *Abscissa*: time from the onset of the experiment (hours); *ordinate*: difference between the number of cells in the left and right halves of the well related to the total number of cells in the well (percent). *1* and *2* — experiment 1; *3* and *4* — control 1 (see M a t e r i a l s and M e t-hods)
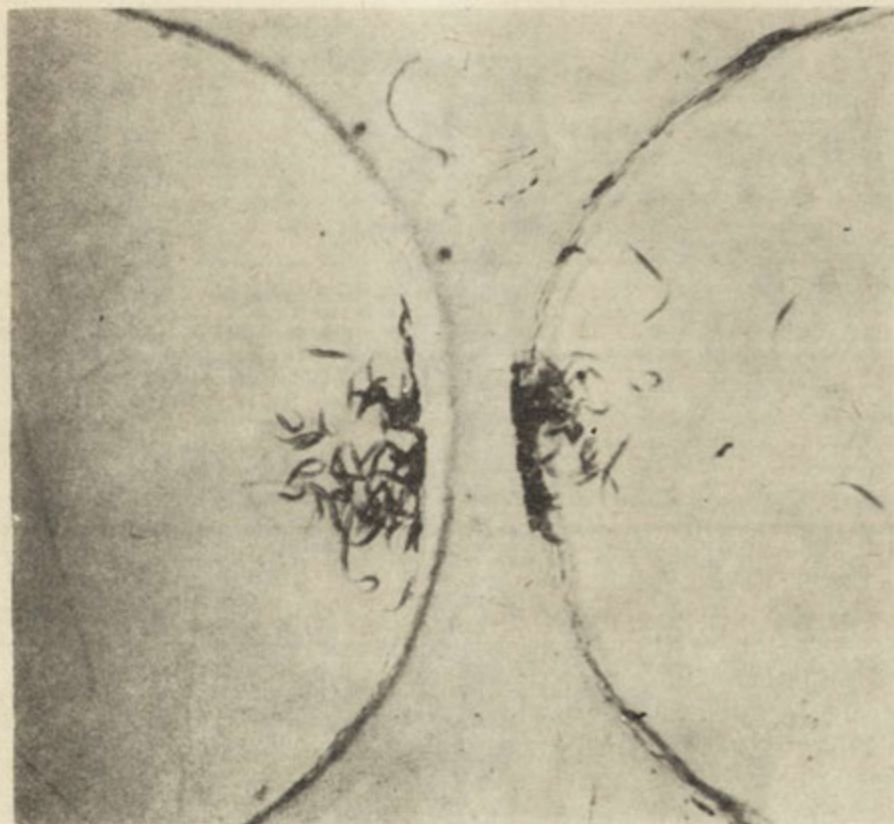
Fig. 3. Uneven distribution of cells of mating types I (left) and II (right) in depressions 2 h after the onset of the experiment

In Experiment 2 CFF of complementary MT ciliates was placed into the neighbouring well vs. cells of a clone under test. In all possible heterological combinations also soon after the onset of the experiment, cells were seen accumulated near the agar isthmus, separating the wells (Fig. 4, curves 1 and 2). But by the end of the second hour or a little later the distribution of cells in the well grew more and more even and 3.5–5.5 h after the onset of the experiment difference in the number of cells between the right and left halves of the well became random. In the control homologous CFF no accumulations of cells were observed (Fig 4, curve 3).

The experiments were repeated many times and yielded similar results. It is known that *Dileptus anser* of all the three MTs excrete gamones autonomously to the surrounding medium: CFF of vegetative cells from most of the clones reduce the waiting period prior to conjugation (Afon'kin and Skovorodkin 1987a, b). It also induces conjugational divisions and/or selfing in ciliates of complementary MTs (Tavrovskaya 1974). Therefore, we assume that the uneven distribution of ciliates in the wells is due to the action of heterologous gamones.
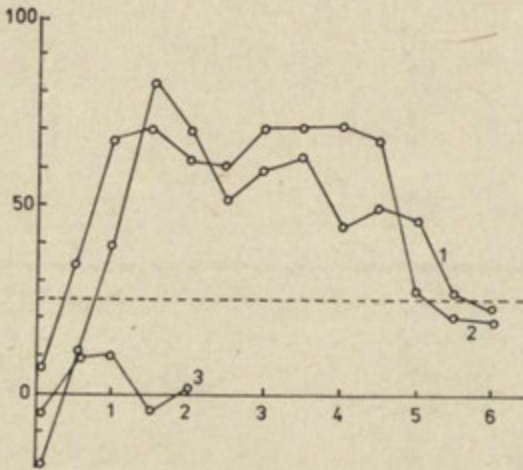
Fig. 4. Experiment 2. An example of uneven distribution of cells under the influence of cell-free culture fluid of clone No. 11. Designations are the same as in Fig. 2. *1* and *2* — experiment 2 (two heterologous CFF of clones Nos. 20 and 12); *3* — control 2

It is still uncertain whether the effect is the consequence of taxis or kinesis. This question has been previously discussed in detail (Afon'kin and Yudin 1986). But whatever the mechanism of *D. anser* response to heterological gamones it must draw closer together cells of complementary MTs to provide for subsequent conjugational interactions.

The uneven distribution of *D. anser* cells in the gradient of heterologous gamones may be used as a rapid and convenient bioassay for the ability of the cells to excrete gamones to the medium.

Further we applied this approach to analyse the spectrum of gamones excreted by clones with genotypes varying in *mat* locus. It is known that clones of *Euplotes raikovi* heterozygous in *mat* locus excrete two types of gamones in unequal proportion, one gamone being the major and the other, the minor (Luporini and Mi-
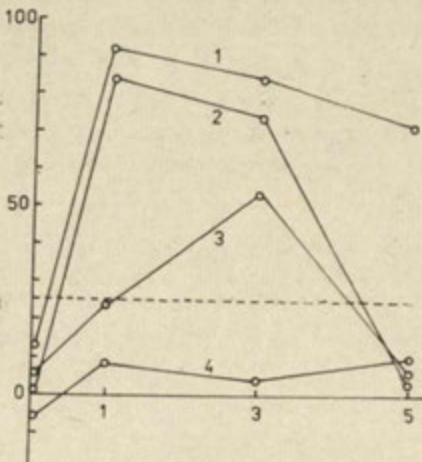

Fig. 5. Distribution of cells from clone No. 11 in the well under the influence of a mixture of homologous and heterologous cell-free culture fluids of clone No. 20. Designations are the same as in Fig. 2 *1, 2, 3* and *4* corresponding to the dilution of heterologous cell-free culture medium 1:4, 1:8, 1:16 and 1:32 respectively

celi 1984). To determine the sensitivity level of our assay, tests were made of artificially composed mixtures of different CFFs. Clones Nos. 11, 20 and 12 and their CFFs were used. Experiments were performed for the most part in the same fashion as Experiment 2 (see Material and Methods) with one exception that a heterologous medium placed into one of the two wells was not "pure" being diluted with CFF of a clone placed in the second well. So, for example, cells of clone No. 11 were put in one well and a mixture of CFFs of clones Nos. 20 and 11 or 12 and 11 (diluted as 1:2) in the neighbouring one. All the six possible combinations were analysed.

The experiments showed that the presence of any heterologous gamone in the culture medium was detectable even on its dilution 1:16 with CFF of a detector clone (Fig. 5). With increase of dilution up to 1:32 no uneven distribution was observed.

Clones obtained from the crosses between test-clones or from induced selfing within them were subjected to an assay for their ability to excrete gamone (Table 1). The genetic experiments enabled us to identify their genotype in *mat* locus and to derive from them clones of a known genotype (Table 1). No clones obtained from the crosses MT I×MT III were used for the experiment since, according to our genetic data, in this case the rule of serial dominance of alleles in *mat* locus was not met. The nature of these deviations is being studied.

Cells of clones Nos. 34, 35, 40, 49 and 50 after maturation were made to encyst and then were preserved as cysts at +10°C for 3 months. Before the onset of the experiment excystment was induced. The test of excysted cells with standard

Table 2

The results of bioassay of clones of known genotype in *mat* locus for gamone excretion

Mating type	Clone tested	Response of test clones		
		No. 11	No. 20	No. 12
I	34	−	+	+
	35	−	+	+
	49	−	+	+
	50	−	+	+
II	40	+	−	+
	56	+	−	+
	57	+	−	+
	61	+	−	+
	65	+	+	−
III	8	+	+	−
	28	+	+	−

Plus — the statistically significant uneven distribution of test-clone cells in the well; minus — the even distribution. Each pair of clones has been tested twice

clones showed that their MT remained unaltered (Afon'kin and Skovorodkin 1987).

All the above clones were tested in pairs with three clones Nos. 11, 20 and 12 used as testers. The depressions with cells were looked through during 5 h beginning from the onset of the experiment. The results of the assay presented in Table 2 may be summarized as follows: when testing clones of a certain MT with a test clone of the same MT we have never detected any deviation from the even cell distribution in the latter clone. The observations show that none of the clones tested have excreted a mixture of gamones to the culture medium.

Discussion

As in the overwhelming majority of ciliates, in *D. anser* the clones are classified as different MTs when conjugating pairs are formed among mixed cells of the clones. It goes without saying that in this case the absence of spontaneous selfing in original clones is implied and checked. Using three clones of different MTs, by means of this standard criterion we succeeded in attributing any sexually mature clone to one of the three MTs first described for *D. anser* by Tavrovskaya (1979). Thus we could ascribe one of the three MTs to each of the numerous clones from various crosses (Yudin and Afon'kin 1987).

On the strength of these experiments a hypothesis was advanced that in *D. anser* the MT is controlled by three alleles with serial dominance in one locus called by analogy *mat* locus (Yudin and Afon'kin 1987).

It must be emphasized here that up to this moment there was practically no information about the very existence, excretion and the spectrum of gamones in clones isolated from nature or produced in the laboratory.

The experiments with clones Nos. 11, 20 and 12 (MTs I, II and III respectively) provide support for the result obtained by Tavrovskaya. Using CFF she showed that each of the three MTs in *D. anser* excretes at least one specific kind of gamone.

The hybridological analysis indicates that all the three clones are homozygous in *mat* locus. The production of clones that are heterozygous in that locus by origin enabled us to investigate the spectrum of gamones excreted by the clones. The results show that in all cases clones heterozygous in *mat* locus release only one kind of gamone corresponding to MT of these clones. Moreover, clones classified with one and the same MT issuing from the classical test of pair formation in mixtures of clones, excrete to the medium only one kind of gamone (from three possible) irrespective of their genotype in *mat* locus.

It is obvious that the above conclusions are determined by the sensitivity of our bioassay. It must be stressed out that (1) the cell concentration in agar and plastic wells was approximately the same (500–700 cells/ml) for two clones under

comparison, and, hence the gamone concentration in two media was similar. (2) *D. anser* cells excrete gamones continuously, and, therefore, the amount of latter in agar wells with ciliates should be constantly replenished. But in wells with CFF mixtures it must be reduced due to diffusion.

In this context, the level of sensitivity established in experiment with CFF mixtures may be considered to be about of the same order as in experiments with pairs of clones under test. Our results and literature data permit the conclusion that in *D. anser* as in some *Euplotes* species, the formal genetics of *mat* locus reflects in essence the inheritance by ciliates of the ability to synthesize and excrete certain kinds of gamones.

From this viewpoint, clones of one species in gamone-excreting ciliates should be classed as different MTs once the spectra of excreted gamones are different. Assuming that a certain ciliate species has N gamones, the number of its MTs would be $(N+1)N/2$ provided co-dominant inheritance of alleles in *mat* locus (the case of *Euplotes*), or N provided their dominance (the case of *Dileptus*).

The number of possible "spectra" from N gamones would have been much greater had the cells been able to excrete 3 or even 4 different gamones. But it is unlikely because of limitations dictated by diploidy.

In this connection, most intriguing are the relationships between gamones and their receptors. Two assumptions may be at the minimum made. The ability to produce one or another kind of gamone might be either closely associated with the spectrum of expressed receptors (the cell has no receptors to its "own" gamones) or there is no such connection at all. In the last case all types of gamone receptors should be present at the cell surface. However, the signal from a receptor to its "own" gamones is somehow blocked, possibly due to imprinting during maturation of the clone (Luporini and Miceli 1984). No matter what the reception mechanism of heterologous gamones may be, one point must be of special interest: had cells of a clone lost all their gamone receptors (e.g., through mutation) but retained their ability to excrete gamones, such a clone might have been, none the less, classified as a definite MT because it could induce selfing in complementary clones when they were mixed.

Such considerations and our own experimental results emphasize the leading role of gamones in defining MT in gamone-excreting ciliates.

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Low Temperature Induces Polyploidization in Dividing *Amoeba proteus*

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Synopsis. The process of mitosis is blocked in one third of the dividing *Amoeba proteus* as a result of the transfer of the cells from $+25°C$ to $+4°C$. The greatest part of such amoebae yield clones after gradual increase of temperature in the culture medium. Measurements of the nuclear DNA content show that such kind of cells and clones that grown from them are polyploid. The role of low temperature as a factor that may induce polyploidization of amoebae in nature is discussed.

There is an idea that the low temperature does not induce any change in the cell genetic apparatus (Aschwood-Smith and Friedmann 1973, Aschwood-Smith and Grant 1977). At the same time, the results obtained recently by cryobiologists and geneticists confirm the Kol'cov's hypothesis that the decrease in temperature may enhance the mutation process (Kol'cov 1938). Cold-induced genetic alterations have been described for organisms of different levels of organization (Birkina 1938, Smith and Corvin 1971, Zavol'naja 1971, Jadin et al. 1976, Sytnik et al. 1980, Calcott and Gargett 1981, Nejfah et al. 1984).

The polyploidization may also be caused by hypothermia. In modern genetic literature this kind of hereditary changes are classed with genomic mutations expressed as alteration of the chromosome number or the chromosome set. In 1890 the Russian botanist I. I. Gerasimov produced cells of the green alga *Spirogyra* with hereditarily enlarged nuclei through incubation of metaphase cells at $+4°C$ (Gerasimov 1890). Those were the first experimentally obtained polyploid forms of plants. For a long period of time up to the discovery of mitotic poisons, the treatment with low temperature was a wide spread method for inducing polyploidy in plant species. More than once, however, attempts were made to use cold for obtaining polyploid forms of fishes (for references see Morris 1984).

In this paper it has been shown that low temperature ($+4°C$) induces polyploid cells and clones in free-living amoebae when dividing cells are rapidly cooled. Such clones are found to be viable. The probability of realization of this process in nature is discussed.

Materials and Methods

Two *Amoeba proteus* strains (War and Bor) were used for the experiments. The strains were maintained in a collection of the Laboratory of Unicellular Organisms (Institute of Cytology, Acad. Sci. USSR). Strain War was brought by Dr. L. N. Seravin from the Nencki Institute in Warsaw, Poland in 1968. Strain Bor was taken from a lake near the biological station "Borok" in 1974.

The culturing and cloning of amoebae were performed as described previously (Afon'kin 1986 a, b). We used three kinds of cell samples — nonsynchronized, partly synchronized and synchronized ones. The obtaining of samples, isolation of nuclei and measurements of the nuclear DNA content were also described in our previous publications (Afon'kin 1984, 1986 a, b).

It should be reminded there that the DNA content was measured cytofluorimetrically and then expressed in arbitrary units. The fluorescence of the tetraploid G_2 nuclei of rat hepatocytes was assumed to be a unit (13.2 pg). As shown by the measurements of the DNA content in the nuclei from amoebae of one strain fixed on different preparations and stained at different periods of time, the mean DNA content may vary significantly. To render the comparison of the DNA amount for different samples more reliable, we correlated data obtained for nuclei fixed on the same slide and thus stained simultaneously.

To measure the nuclear area isolated and fixed nuclei were stained with toluidine blue and $Na_2B_7O_4$ during 3 min. Thereupon the slides were washed off with distilled water. The nuclear areas were measured by means of an automatic TV device "Ismeritel-1", and were expressed in arbitrary units. In this paper 1 arb. unit is 7.3×10^{-5} mm^2.

For evaluating the cloning efficiency 60 amoebae were cloned in a microplate (Afon'kin and Yudin 1986) at $+25°C$. The culture medium contained food organisms throughout the experiment. After 72 h the proportion of subclones in which at least one division took place was estimated. This value was assumed as a cloning efficiency. When calculating the generation time, the cloning efficiency was taken into consideration (for detail see Afon'kin 1986 a, b).

The original ploidy level in *A. proteus* is not yet known. As in the previous paper, it was arbitrarily taken as diploid. The clones in which the nuclear DNA content was in consistency with the hypothesis of two- and four-fold increase of the control level were considered to be tetraploid and octoploid.

Results

Producing War Polyploid Clones

Before the onset the experiment four independent samples of dividing amoebae were taken from a mass culture of the strain War cells. Each sample consisted of 50 amoebae. During next 30 min ($+25°C$) $83.0 \pm 2.3\%$ of the amoebae completed their division, $11.5 \pm 2.8\%$ of the cells were binucleate, and $5.5 \pm 0.9\%$ mononucleate. The proportion of nonviable nondividing (upon cloning) War amoebae was 1.7% (three independent samples, 60 amoebae in each). These facts lead to the as-

sumption that the group of nondivided mononucleate amoebae in the control con-
sisted of cells uncapable of completing their division and cells erroreously taken
for dividing ones.

The first series of experiments was performed to define whether polyploid cells
arise after cold treatment, and, if it is true, what is their proportion.

Small groups of cells (5–10 amoebae) dividing at +25°C were transferred from
the mass culture to the cooled (+4°C) culture medium. The cytokinesis was com-
pletly blocked at that temperature. All in all three independent samples of amoebae
(126, 95 and 125 cells) were produced by this method. After 24 h the medium was
gradually warmed (+25°C) and food was supplied. Only a very small number of
amoebae died (7%). The surviving amoebae did not divide at least during the next
24 h. By the end of this period, the cells were destroyed and their nuclei were tran-
sferred onto the slide.

After the cold treatment of dividing amoebae 36.6±0.5% of the cells were
found to be mononucleate what is 6.6-fold higher than in the control. We suggested
that karyokinesis of such cells was blocked by low temperature. To verify the hy-
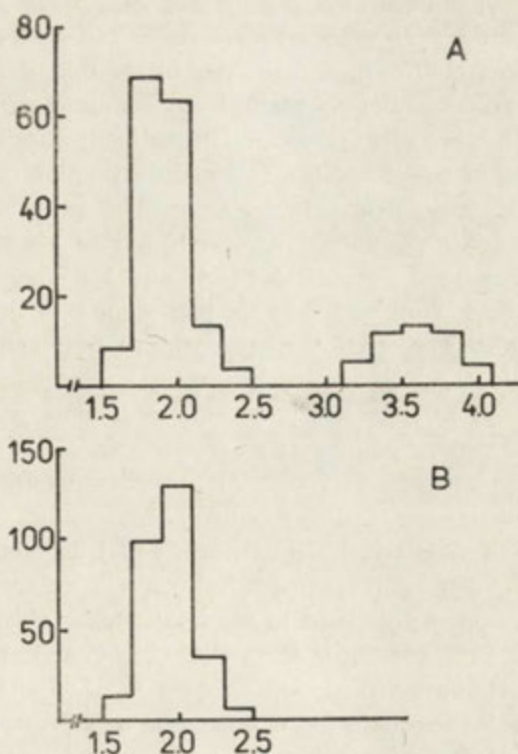pothesis the nuclear DNA content in the last group of cells (120 amoebae without

Fig. 1. Distribution of values for DNA content in War cells after cold treatment of dividing amoebae.
Abscissa: DNA content (arb. units), *ordinate*: number of nuclei. A — experiment, B — the control

dying) was measured. As we have expected the distribution of obtained values included two groups of nuclei with smaller and larger DNA amount (Fig. 1). All mononucleate cells (45 amoebae) had a 1.87–fold increased DNA content as compared to the binucleates (75 amoebae, 150 nuclei).

The DNA content in the nuclei of three independent samples of synchronized cells that were cultured with food after the completion of cytokinesis during 24 h ($+25°C$) were used as a control. The mean values (1.92 ± 0.02; 1.96 ± 0.02; $1.94\pm\pm0.02$ arb. units) did not differ significantly and so all figures were united into a single sample (Fig. 1 B). The second order mean value did not differ from the mean level of the DNA content in binucleate cells in the experiment (1.92 ± 0.02 and 1.92 ± 0.01, respectively).

It should be noted that among three control samples there were eight cells (2.8%) with greatly increased DNA content (3.2–4.4 arb. units). These values statistically differed from the rest of the figures.

So we have shown that in 36.6% of the cases the low temperature blocks the cytokinesis of dividing amoebae and in such cells the nuclear DNA content increases as a result.

The second series of experiments was performed in order to investigate the proportion of viable polyploid cells. For this purpose cells obtained by cold were cloned. The greatest number of amoebae were viable and gave clones. Overall, 41 (17.1%) amoebae from 240 died, and the rest of the cells started division.

To evaluate the proportion of polyploid clones five nuclei were isolated (without any special synchronization) from each of 120 randomly chosen clones and their DNA content was then measured. The obtained distribution of mean values was represented by two groups of figures. In one group (87 clones) the means were between 1.09 and 1.66 arb. units, and in the other (33 clones) between 2.38 and 3.63 arb. units. Two second order means were 1.47 and 3.01 arb. units, respectively. The ratio between them being 1:2.04. So the proportion of viable polyploid clones was 27.5%, or, in other words, 3/4 of all cells with cold-blocked karyokinesis were found to be capable of giving clones.

To evaluate the DNA content in polyploid clones more precisely, three independent samples of 5 partly synchronized nuclei were picked out from each of them. In this case the samples from three clones grown from binucleate amoebae with cold-blocked cytokinesis were used as a control. Three second order means in the control (1.49 ± 0.05; 1.47 ± 0.05; 1.52 ± 0.06 arb. units) did not statistically differ from one another, and thus all control values were united into a single sample. The third order mean was compared to the mean values obtained for polyploid clones. The nuclear DNA content in them was 2.03 ± 0.6-fold greater than in the control. The minimal and maximal correlations made 1.95 and 2.10 respectively. Taking into account errors of the mean values, we may prove that in two above cases the DNA content in polyploid clones was increased two times.

Thus we managed to show that quick cooling of dividing amoebae down to

+4°C is responsible for the appearance of a great number of cells with blocked mitoses. Such amoebae may produce viable tetraploid clones. It is not improbable that clones with a higher ploidy level might be obtained by the method described. The following fact provides another evidence in favour of the suggestion: two strikingly large cells were picked out from War mass culture subjected durably to low temperature (the experiments are not discussed in this paper). Clones War-34 and War-35 were then grown from them. The measurements of the DNA content showed that the clones are octoploid as compared to the mass culture amoebae (Table 1).

Table 1

Nuclear DNA content in clones War-34 and War-35

Clone	The second order mean of DNA content, $\overline{X} \pm S\overline{x}$, arb. units
War (control 1)	1.82 ± 0.07
War (control 2)	1.84 ± 0.10
War-34	7.24 ± 0.74
War-35	7.82 ± 0.50

All second order mean value were obtained through analysis of 5 independently obtained partly synchronized samples. Each sample included 5 nuclei. Two clones grown from a War mass culture were used as a control.

We known nothing about the origin of such cells in War mass culture but the very fact of their presence indicates that the possibility exists of inducing viable octoploid clones in War amoebae.

To consider the potentialities of a two-step polyploidization by cold in *A. proteus* and clear up the strain specificity of such process, the following experiments were performed with Bor amoebae.

Producing Bor Polyploid Clones

As with the War cells, small groups of dividing Bor amoebae were transferred from mass culture (+25°C) to the cold medium (+4°C). After 3–4 h the medium was gradually warmed up to +25°C. The cytokinesis in this case was also blocked. Clones were then grown from the amoebae cells. All in all 150 clones were obtained by this method. Since at this stage of our work we did not undertake the task of evaluating the proportion of polyploid clones, for further research only a part of produced clones was chosen. We selected clones that induced only the large amoebae.

During the next week we made up 5 independent samples, each including 5 non-synchronized nuclei isolated from the chosen clones. The nuclear area and the DNA content were measured and two second order means of these values were calculated for each clone. Five clones grown from a Bor mass culture were used as a control.

The results show that 25 clones grown from cold-treated amoebae formed one isolated group according to their nuclear size and the DNA content (Fig. 2). All dots denoting the DNA content localize between the two-fold increased limits calculated for the control. The third order means values of the DNA content in
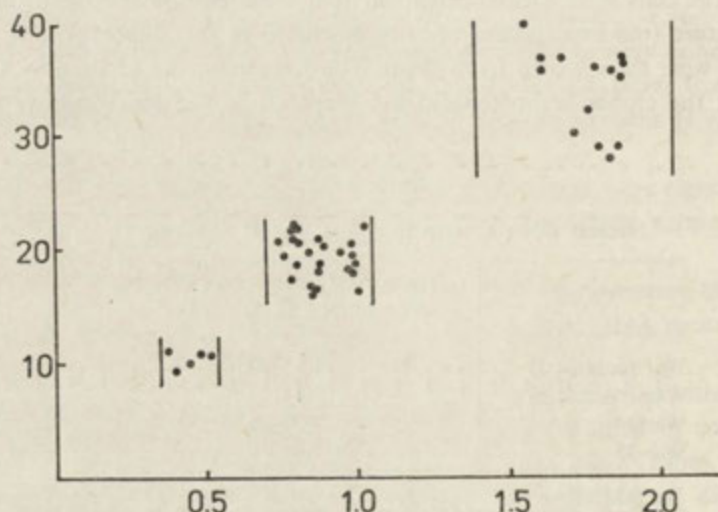

Fig. 2. Nuclear size and their DNA amount in clones grown from cold-treated dividing amoebae. *Abscissa*: DNA content (arb. units), *ordinate*: nuclear area (arb. units). Each point represents two second order mean values (see the text). The left group of points is the control. The pairs of vertical lines show two- and four-fold increased limits of DNA variation in the control

two groups of dots are 0.45 ± 0.02 and 0.87 ± 0.02 arb. units, the ratio being $1:1.93$. With the errors of the means it is not of variance with the hypothesis of two-fold increased DNA content in 25 clones.

For obtaining octoploid clones by this method a mass culture was grown from a randomly selected tetraploid Bor clone (Bor-7), thereupon the whole procedure was repeated. As a results, 14 clones were obtained (the right upper group of dots in Fig. 2), the DNA amount in them being increased 3.93-fold as compared to the control values.

All 14 dots lie within the four-fold increased control limits (Fig. 2). This fact and the errors of the means as well permit the assumption that the DNA content of these clones was four-fold increased. In other words, they were truly octoploids.

Cloning Efficiency, Generation Time and Stability of Polyploid Clones

As shown previously, the mean generation time of polyploid cells in *A. proteus* clones obtained by colchicine injections into dividing amoebae remain unchanged but the cloning efficiency decreases (Afon'kin 1984). This is also true for polyploid clones picked out from mass cultures (Afon'kin 1986a).

The polyploid clones obtained by cold treatment form no exception to this rule. The cloning efficiency in two of the five randomly chosen tetraploid War clones was lesser than in the control (Table 2). The mean generation time of all five clones did not differ from the control values (Table 2).

It is known that the ploidy level of polyploid amoebae may spontaneously decrease as a result of a stepwise depolyploidization of cells (Afon'kin 1986b). This phenomenon was also detected in several clones discussed in the present paper.

Table 2

Cloning efficiency and generation time of tetraploid War clones

Clone	Cloning efficiency, %	Generation time, h
War (mass culture)	98.3	32.6 ± 0.8
War (control 1)	98.3	32.0 ± 0.8
War (control 2)	95.0	31.3 ± 0.8
War-1	96.6	32.2 ± 0.8
War-2	96.6	33.6 ± 0.9
War-7	85.0*	32.4 ± 0.9
War-11	91.6	32.7 ± 1.0
War-20	86.6*	32.8 ± 1.1

Stars indicate cases when the cloning efficiency differs significantly from control values.
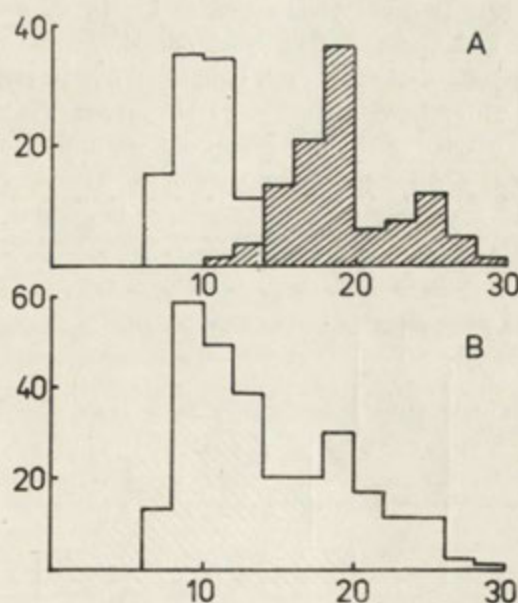

Fig. 3. Distribution of values for nuclear area in nonsynchronized Bor amoebae. *A*, left — mass culture, right — clone Bor-7, *B* — clone Bor-14. *Abscissa*: DNA content (arb. units), *ordinate*: number of nuclei

Table 3

DNA Content in 1 h nuclei in Bor-7 and Bor-14 clones

Clone	Number of nuclei measured	DNA content $\overline{X}\pm S\overline{x}$, arb. units	
		in the sample	second order mean
Bor	28	0.31 ± 0.01	
	20	0.28 ± 0.01	0.29 ± 0.01
	29	0.29 ± 0.01	
Bor-7	35	0.66 ± 0.02	
	19	0.62 ± 0.03	0.64 ± 0.01
	33	0.65 ± 0.01	
Bor-14	15	0.32 ± 0.01	
	19	0.33 ± 0.01	0.31 ± 0.01
	15	0.30 ± 0.01	
	12	0.58 ± 0.03	
	10	0.69 ± 0.02	0.63 ± 0.02
	18	0.62 ± 0.01	

The first indication to the onset of depolyploidization was the increased hetero-
geneity in the size of cells and nuclei in the tetraploid clone Bor-14 (Fig. 3). In this
case the Bor mass culture and tetraploid clone Bor-7 were used as a control.

To confirm the stepwise depolyploidization in Bor-14 we measured the DNA
content in this clone and in Bor-7. The synchronized samples were used for the
measurements. The results show that each sample of Bor-14 may be divided into
two groups of nuclei with a lesser and greater DNA amount (Table 3). For all that
the second order mean value in the first group did not differ from the correspon-
ding control value obtained for the Bor mass culture. The second order mean in
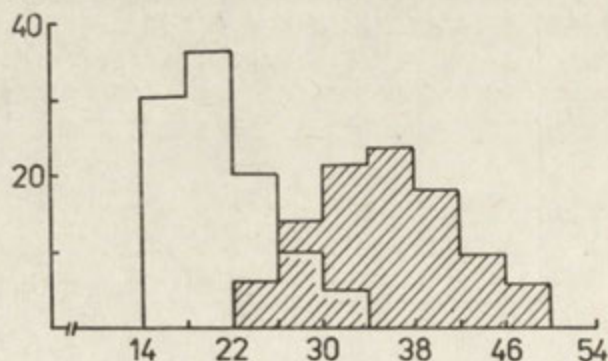the second group did not differ from the corresponding figure in the tetraploid

Fig. 4. Distribution of values for nuclear area in nonsynchronized amoebae of clone Bor$_{octo}$-3
(right) and Bor$_{octo}$-13 (left). *Abscissa*: DNA content (arb. units), *ordinate*: number of nuclei

Bor-7 clone (Table 3). The results show that the clone Bor-14 when measured contained two kinds of cells with a normal and doubled DNA content.

There is an indirect evidence indicating the depolyploidization in one of the octoploid Bor clones. The nuclear area in $Bor_{octo}-13$ clone 3 months after it been obtained did not differ from the value characteristic of tetraploid clones (Fig. 4, cf. Fig. 2, 3). Only one nucleus with the nuclear area 44.4 arb. units was still "octoploid". The nuclear area of the stable octoploid clone $Bor_{octo}-3$ was used as an additional control (Fig. 4). Three months after it had been produced, its nuclear size remain unchanged showing features typical of octoploid clones (Fig. 2).

Our previous experience shows that in the $Bor_{octo}-13$ clone we have case of depolyploidization from octo — to tetraploid level.

Discussion

Free living amoebae survive in a wide thermal range. The multiplication rate decreased and the generation time is enhanced with the lowering of temperature. At $+4°C$ amoebae are not able to divide, the cyto- and karyokinesis are blocked and the DNA is being synthetized very slowly. The influence of low temperature is reversible. After the transfer from $+4°C$ to $+25°C$ the rate of DNA synthesis increases and shortly after amoebae start division (Sopina 1976, Sopina et al. 1982).

In the literature there is an evidence that low temperature blocks cytokinesis in *A. proteus* (Daniel and Chalkley 1933). Rustad (1960) produced a great number of binucleate cells by transferring dividing amoebae to $+6°C$ for 4 h. None the least, he did not investigate mononucleate cells. Roth (1967) found that low temperature was responsible for the depolymerization of microtubules in the mitotic spindle of dividing *A. proteus*. So it appears that molecular mechanism of the low temperature effect is similar to that of mitotic poisons. In fact, polyploid cells of A. *proteus* may be produced by low temperature or by injection of a colchicine solution as well (Afon'kin 1984).

The original ploidy level in *A. proteus* is not known. It may be assumed arbitrarily as diploid. The precise number of chromosomes (several hundreds) in this species is difficult to count both at the optical or electronmicroscopical level (Gromov 1985). That is why the cell polyploidization in *A. proteus* is estimated by the enlarged nuclear DNA content.

Colchicine injections into dividing cells of strain B amoebae in 38% of the cases caused the appearance of tetraploid cells (Afon'kin 1984). The results obtained show that the same portion (36.6%) of War cells with blocked karyokinesis is induced after cold treatment. It is most possible that 36–38% of the chosen "division spheres" are in prophase.

The measurements show that the nuclear DNA content in clones grown from

cells with cold-blocked mitoses is doubled as compared to the control. Therefore, this enables one to regard these as tetraploid. The nuclear DNA amount in cells immediately after polyploidization (24 h at $+4°C$ and 24 h at $+25°C$) was, however, only 1.87–fold greater than in the control amoebae (Fig. 1A). This fact may be explained by the assumption that the ratio "nuclear volume/nuclear surface" may be changed as a result of the karyokinetic block.

Such a change can account for the reduced rate of DNA synthesis. In this case a suggestion may be made that the ratio is gradually normalized with the increase of the nuclear surface.

In natural lakes and ponds free-living protozoans undergo by far crucial thermal shifts. It is not excluded that polyploidization may occur under natural conditions, but for its realization the water temperature that still allows cell division should decrease to a level when mitosis is practically blocked.

In the previous paper a hypothesis was advanced that the process of spontaneous polyploidization and depolyploidization may be a source of genetic variation in the *A. proteus* — agamous organism (Afon'kin 1984, 1986 a, b). It may be supposed that polyploid amoebae have physiological features others than normal cells, as in the case of some plant species (Breslavec 1963, Savčenko 1976). Polyploid clones may be more resistant to extremal factors fulfilling partly the function of cysts (*A. proteus* is not known to have cysts). This hypothesis, however, calls for verification.

Our results and finding of other investigators who studied mutagenous effects of hypothermia show that the low temperature is and active genetic factor that affects cells, free-living protozoans inclusive.

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Uptake Pathway of Lipid Vesicles in *Tetrahymena pyriformis*

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Synopsis. The inhibitory effect of dichloroisoproterenol (DCI) on the endocytotic activity in *Tetrahymena pyriformis* cells has been used to investigate the uptake pathway of cholesterol-lecithin vesicles in these ciliates. The pre-exposure of the cells to 40 µM DCI caused complete block of digestive vacuole (DV) formation in all the tested cells placed in the medium with latex suspension. Preincubation of the cells with DCI inhibited the cell uptake of labelled cholesterol-lecithin liposomes in 70%. Such decrease in the lipid vesicle incorporation by *Tetrahymena* cells in the liposome-containing medium was shown to be reversible as in the case of latex uptake. It is suggested that lipid vesicles enter the *Tetrahymena* cell mainly by cell endocytosis.

Lipid vesicles have been used as model membranes to elucidate the physico-chemical organization of lipid bilayers and to study the interaction of various membrane-active drugs with lipids (Papahadjopoulos and Kimelberg 1973, Bangham et al. 1974, Tyrrel et al. 1976, Gregoriadis 1977, 1980, 1982). The experiments in several laboratories have shown that mammalian cells *in vitro* and *in vivo* will incorporate large numbers of lipid vesicles without cytotoxic effects. Since a wide variety of materials can be entrapped inside vesicles and various glycoproteins, glycolipids and sterols can be inserted into the vesicle membrane, the cellular uptake of liposomes of defined composition offers a potential method for modifying cellular composition and for introducing non-permeable biologically active materials into the cell interior (Papahadjopoulos et al. 1974, Poste and Papahadjopoulos 1976, Poste et al. 1976, Venkatakrishman et al. 1979, Bkaily et al. 1983a, b Fabczak 1986a).

Using lipid vesicles as carriers to introduce materials into the cell cytoplasm, relatively little attention has been given to the kinetics of vesicle-cell interaction and the mechanism by which vesicles might be incorporated into the cell. It is obvious, however, that the use of lipid vesicles as carrier vehicles to transport materials into the cell demands at least some basic appreciation of the mechanism by which

these structures are transposed. It is reasonable to assume that in the cells with en-
docytotic ability the liposome uptake might occur either by endocytosis or/and
by fusion of lipid vesicles with the cell plasma membrane (Poste and Papahadjo-
poulos 1976, Hallett and Campbell 1980).

There has been reported recently that DCI is able to inhibit endocytotic uptake
of food materials in *Tetrahymena* and *Paramecium* cells (Ricketts 1983, Fok
and Shockley 1985, Wyroba 1986). Therefore, the main purpose of this study
is an exploration of the uptake mechanism of lipid vesicles in *Tetrahymena* cells
by use of this kind of drug to alter the endocytotic cell activity. Preliminary report
of these results has been published elsewhere (Fabczak 1986 b).

Material and Methods

Stock culture of *Tetrahymena pyriformis* was grown in 2% proteose peptone medium (Difco)
with 0.1% yeast extract (Difco) at 28°C. The three-day-old cell culture was collected by centrifuga-
tion at 800 g and washed twice with buffer solution (i.e., 1 mM TRIS/HCl, 1 mM $CaCl_2$ at pH 7.2).
Ciliate collecting and washing procedures were carried out in aseptic conditions only. The cell
starvation was applied during 20 h before each experiment at 20°C in sterile buffer.

For determination of liposome number effectively uptaken by *Tetrahymena* cells in the course
of experiment, they were incubated with liposomes containing lecithin, cholesterol, and small amount
of ^{14}C-cholesterol (2 μC/ml, specific activity 57 mCi/mM) with molar cholesterol to lecithin ratio
of one (C:P = 1). Liposomes were prepared according to method of Batzri and Korn (1973),
modified by Fabczak (1986 a). Every 30 min a sample of starved cells was taken out and washed
three times in buffer solution (by centrifugation at 800 g). Then the sample was extracted with five
volumes of chloroform and methanol (3:1 v/v). The organic phase of the cells was removed and
dried under N_2. The sterol radioactivity was determined with the aid of Backman counter.

To measure the inhibitory effect of DCI (Sigma) on the endocytotic activity of *Tetrahymena*
cells, a cell sample was incubated with the drug at concentration of 40 μM for 30 min, washed
in buffer solution and placed for 5 min in latex beads suspension (Difco, 0.8 μm diameter). After
different time periods (from 30 to 150 min) the cell samples were washed, fixed in 2.5% glutaral-
dehyde in phosphate buffer at pH 7.2 and the number of DV per cell was counted under micro-
scope. Other samples of DCI-treated cells were placed in suspension of liposomes with labelled
cholesterol and after different time intervals, as in case of latex suspension, the samples were pro-
cessed for radioactivity (i.e., sterol uptake) determination.

Protein concentration in the cell homogenate was estimated according to method of Lowry
et al. (1951).

Results and Discussion

Data presented in Fig. 1 on the cellular uptake of lipid vesicles with radiola-
belled cholesterol by cells of *Tetrahymena* show that vesicles can be easily incorpo-
rated into the cell interior. The general uptake kinetics at room temperature is
characterized by rapid uptake of liposomes occurring within the first 2 h and reach-
ing a plateau. The total amount of labelled cholesterol incorporated into cells at-
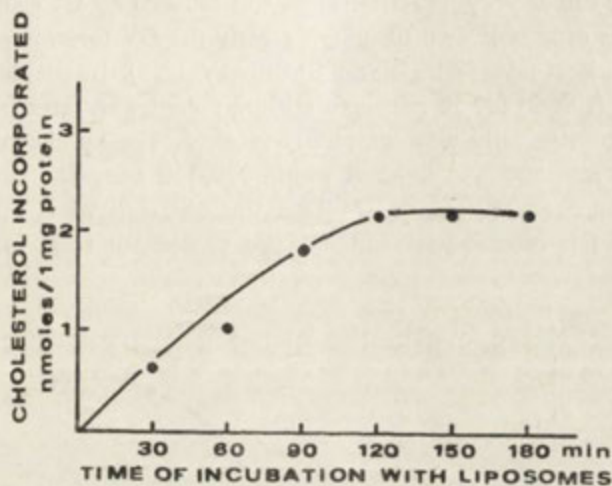
Fig. 1. Uptake of liposomes (C:P = 1) by *Tetrahymena pyriformis*. Vesicle uptake was determined at the indicated time intervals by measuring the radioactivity of ^{14}C-cholesterol

tained about 2 nmoles of cholesterol during 2 h incubation with liposomes at C:P ratio of one. That gives 4 nmoles of lipids per 1 mg of protein of *Tetrahymena* cells.

The data correspond well with those obtained by Papahadjopoulos et al. (1974) in study of uptake phenomena in mammalian cultured cells. The mammalian cells are able to incorporate from 0.2 to 6 nmoles of lipids per 10^6 cells with similar uptake kinetics as in the case of protozoan cells. Therefore, it seems to be true that uptake pathway in ciliates is approximately the same as in mammalian cells. Poste and Papahadjopoulos (1976) suggest that the cells can incorporate the lipid vesicles via fusion and endocytosis mechanisms.

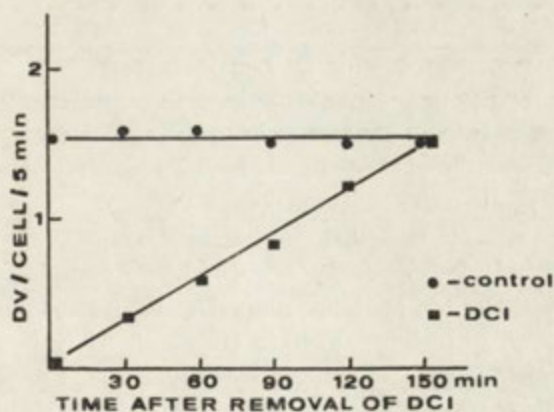To elucidate which of the mentioned above uptake pathways predominates,

Fig. 2. Effect of DCI on the rate of DV formation. The cells were preincubated in 40 μM DCI for 30 min before being fed latex beads for 5 min

the endocytotic cell activity in *Tetrahymena* was reduced by DCI added to the medium, since this drug is known to inhibit greatly the DV formation process in *Tetrahymena* (Ricketts 1983, Fok and Shockley 1985). In the cells pre-exposed to 40 µM DCI (Fig. 2) for 30 min before being pulsed with latex beads for 5 min, the DV formation initially was completely blocked. The uptake recovery process began after 30 min and was finished within 120–150 min. The similar inhibition of the endocytotic activity at 40 µM DCI was observed when cells were placed in suspension of liposomes with radiolabelled cholesterol (Fig. 3). On the other
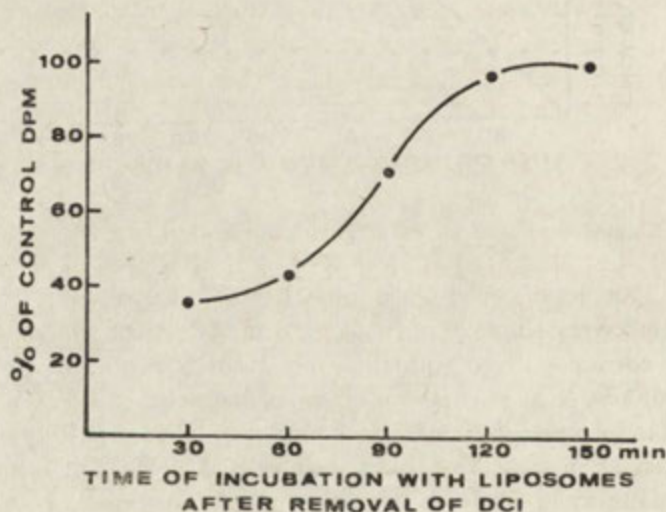

Fig. 3. Uptake of liposomes by *Tetrahymena pyriformis* after removal of DCI. Ciliates were preincubated with 40 µM DCI for 30 min before being washed, then pulsed with liposomes (C:P = 1). For each time point the incorporation of radiolabelled liposcmes into DCI-treated cells was expressed as percent of control

hand, *Tetrahymena* pretreated with DCI and incubated with liposomes demonstrated only 30% of the radioactivity of the DCI-untreated cells, indicating that the cell endocytic activity was reduced considerably. As in the case of latex uptake the liposomal material uptake inhibition by DCI was reversible and the initial endocytotic activity was restored within 2 h.

The obtained results reveal that phospholipid vesicles may be incorporated into the cell of *Tetrahymena* not only through endocytotic pathway, although this type of activity in the cell is predominating. The similar conclusion has been put forward by Post and Papahadjopoulos (1976), showing that 3T3 cells can incorporate lipid vesicles almost exclusively by endocytosis. The fluid vesicles endocytosis by liver cells *in vivo* has been concluded on the basis of electron microscopic

data obtained by Rahman and Wright (1975). Korn et al. (1974) have also reported endocytosis of lipid vesicles by *Acanthamoeba*.

Therefore, the presented data are the further evidence to support the previous suggestion that by manipulating the composition of lipid vesicles it may be possible to achieve important and convenient method of loading the cell interior with wide variety of materials. The phospholipid vesicles "that enter cells by endocytosis would be directed into the lysosomal apparatus with subsequent breakdown and release of vesicle contents", thereby changing cellular composition (Poste and Papahadjopoulos 1976).

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Endocytotic Ability of Starved *Paramecium bursaria*

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Synopsis. Food vacuoles formation rate in *Paramecium bursaria* depending on the duration of starvation has been studied. The ability to take up the edible and inedible particles was compared. Latex beads and carmine suspension were treated as inedible material, while carmine suspension with bovine albumin fraction V and carmine with bacteria *Klebsiella aerogenes* were treated as edible. It was found that the rate of endocytosis decreases proportionally to the duration of starvation and depends on the character of particles used for feeding the ciliates. The presented results lead to the conclusion that edible material is taken up more readily than inedible, by the long-lasting starved *Paramecium*.

Paramecium has been known as a ciliate which takes up indigestible particles as readily as bacteria or other edible particles. Its food is obtained from bacteria and other nutritive materials by the process of endocytosis resulting in the formation of food vacuoles. In this process the cytoplasmic membrane of the cytostome is highly involved. In most cases the number of food vacuoles formed at a given time is related to the concentration of suspension in the surrounding medium (Railkin 1981, Wasik 1983, Wasik and Sikora 1984b). However, in the case of *Tetrahymena*, even in the first 10 min of feeding this relation is not proportional (Nilsson 1972, 1976), Also other factors influence the food vacuoles formation. In *Tetrahymena*, for instance, there should be present a sufficient concentration of particles to be incorporated into all food vacuoles formed (Chapman-Andresen and Nilsson 1968), and a given cell should not be in the process of cell division (Mast 1947, Nilsson 1979). The rate of food vacuoles formation in *Tetrahymena* depends also on the duration of starvation (Ricketts 1971 a, b, Nilsson 1972, Rothstein and Blum 1974). The fed cells rapidly take up edible and inedible particles, while long starved ciliates take in only digestible ones (Seamen 1961, Ricketts 1971 b).

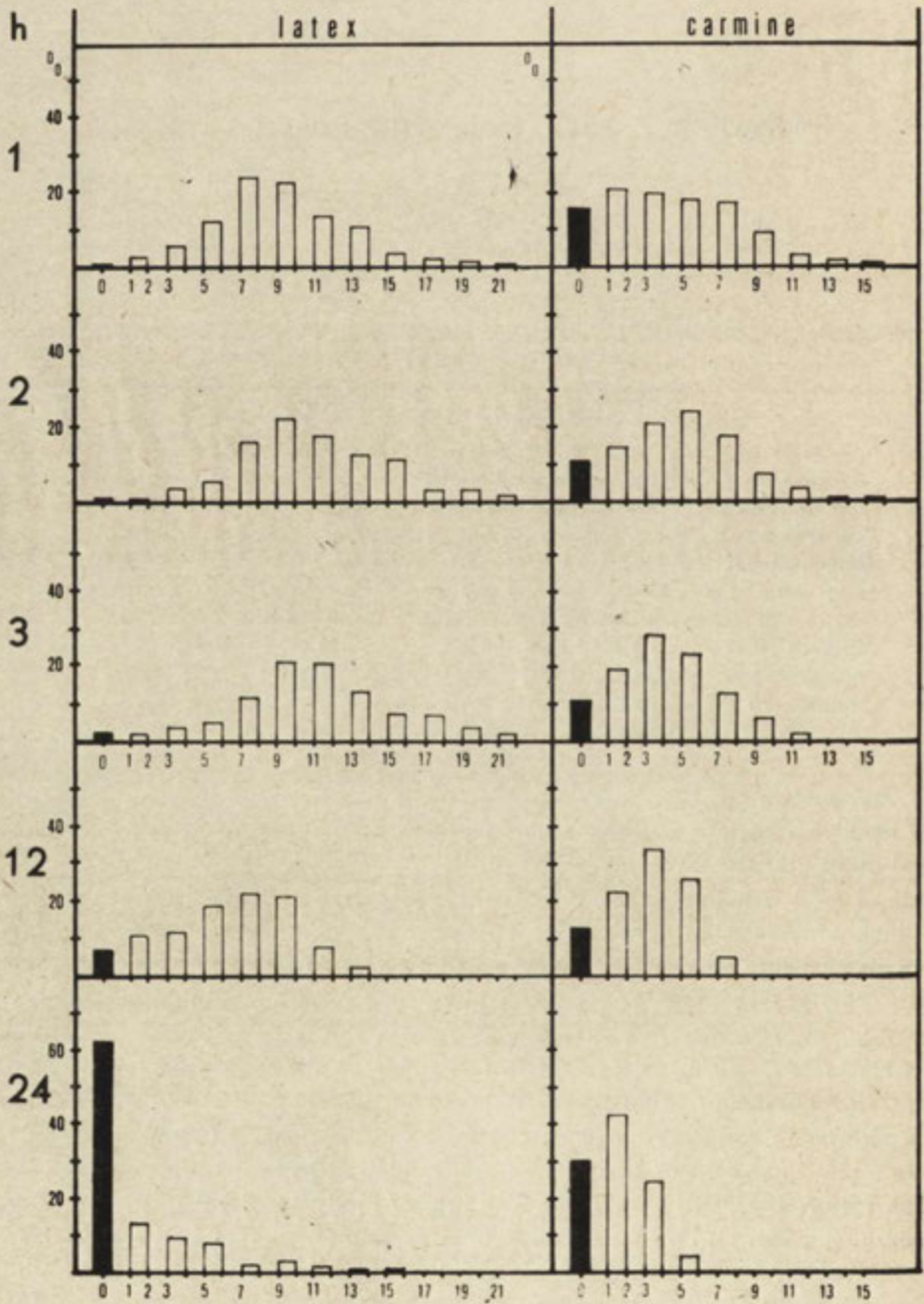Fig. 1. Food vacuoles formation rate of *Paramecium bursaria* cells during 3 min exposure to latex beads, carmine particles, carmine particles with bovine albumin and carmine particles with bacteria *Klebsiella aerogenes* after 1, 2, 3, 12, 24 h starvation time in mineral maintenance solution, with the exception of carmine with bacteria, where no data are available for 3 and 12 h starvation effect.
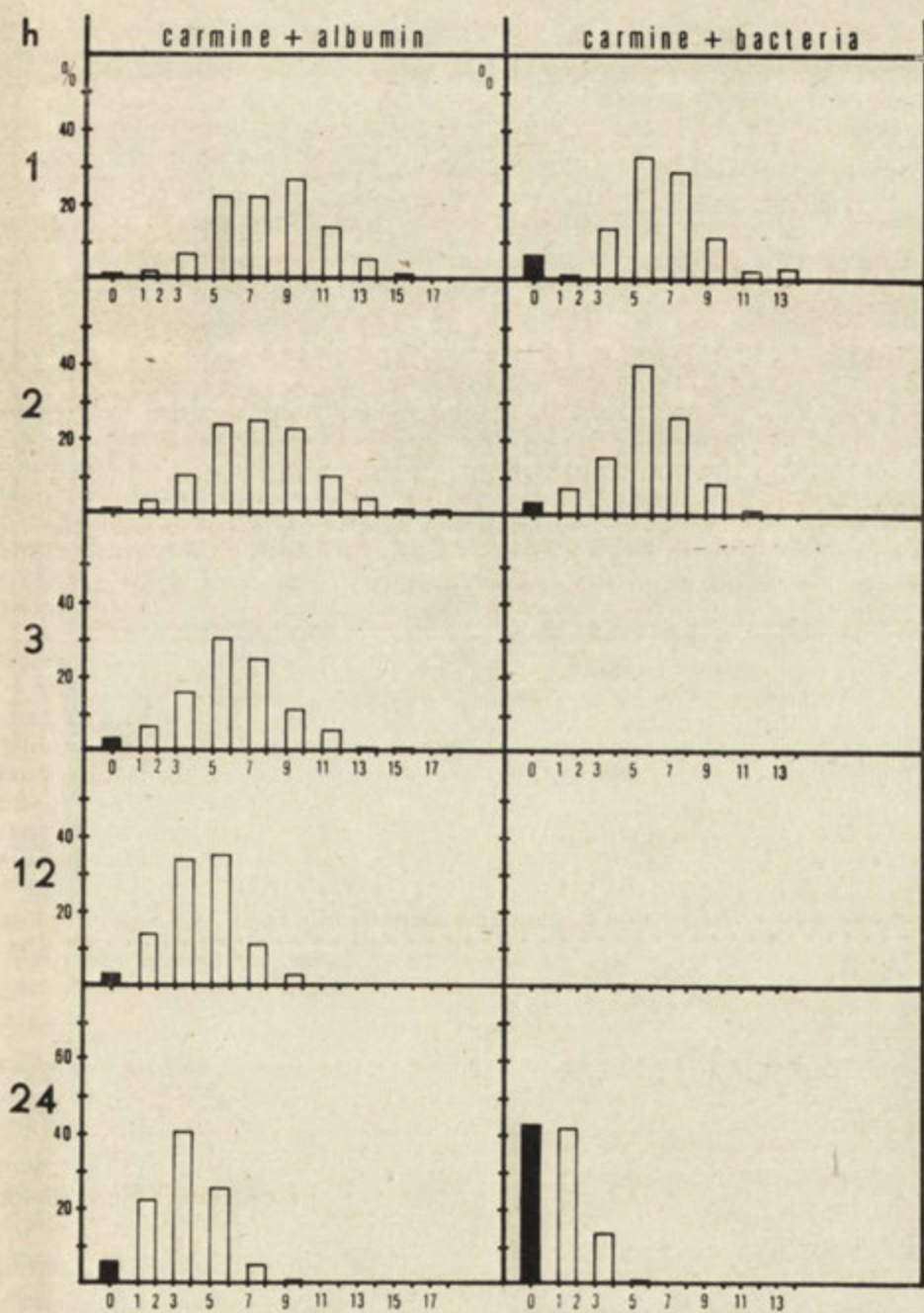
Results expressed as a number of food vacuoles per cell (*abscissa*) formed in the percentage of cells (*ordinate*). Each block represents cells which formed 1 and 2, 3 and 4, 5 and 6 ... and so on food vacuoles, excluding the solid block representing only one class of cells which do not form vacuoles at all

Since *Paramecium* amd *Tetrahymena*, being *Hymenostomata*, are related to each other, it was reasonable to suppose that they would response similarly if exposed to starvation.

Therefore the aim of this paper was to establish whether the duration of starvation in *Paramecium bursaria* would affect the rate of food vacuoles formation. Another purpose was to determine whether the differences in the rate of uptake of edible and inedible particles found in *Tetrahymena*, are present also in *Paramecium bursaria*.

Materials and Methods

The ciliate used in the experiments was *Paramecium bursaria*, devoid of intracellular *Chlorellae*, grown in darkness. The cells were cultured at room temperature ($19 \pm 2°C$) on Scottish grass medium (Sonneborn 1970) inoculated with *Klebsiella aerogenes*. Cells were purified by filtration and concentrated by centrifugation at $300 \times g$ for 1.5 min. Then they were washed with mineral maintenance solution (M.S.) containing 5 mM Tris-HCl buffer (pH 7.25) with 1 mM KCl and 1 mM $CaCl_2$ (Wasik 1983), used as a starvation medium. This solution was filtered to remove particulate material. The density of paramecia used in the experiments was 1000 cells/ml determined, after fixation of 0.2 ml sample mixed with 1.8 ml of 3% glutaraldehyde in cacodylate buffer (pH 7.3), by using the counting camera.

Before experiments the paramecia were starved for 1, 2, 3, 12, and 24 h in mineral maintenance solution.

For the estimation of the rate of food vacuoles formation, the starved cells were fed for 3 min with polystyrene latex beads of 1.04 μm diameter (Polyscience Inc.) in a final concentration of 10^8 particles/ml, carmine (B.D.H.) suspension (particles of about 1 μm in diameter and 10^8 particles/ml concentration), or carmine with 0.05% bovine albumin fraction V (Biomed — Kraków), or with bacteria *Klebsiella aerogenes* (10^8 cells/ml) grown on agar and suspended in M.S. Then the paramecia were fixed with 3% glutaraldehyde in cacodylate buffer (pH 7.3). The rate of food vacuoles formation was estimated according to Nilsson (1979) and Wasik (1983).

Experiments were performed at room temperature ($19 \pm 2°C$).

Results

The purpose of our study was to estimate the influence of starvation time on food vacuoles formation rate in *Paramecium bursaria*, and to check up the probable differences in edible and inedible particles uptake.

Latex beads and carmine suspension, as examples of inedible particles, were used. The same carmine particles with bovine albumin fraction V, or mixed with bacteria *Klebsiella aerogenes*, were used as edible.

Figure 1 shows the changes in the number of food vacuoles formed during 3 min exposure of *Paramecium bursaria* to latex, carmine, carmine with albumin and carmine with bacteria for 1, 2, 3, 12 and 24 h of starvation in M.S. Table 1 demonstrates the mean values of food vacuoles formed by paramecia depending on the duration of starvation and on exposure to edible or inedible particles.

Table 1

Mean values of food vacuoles formed by *Paramecium bursaria* fed with diferrent types of parti-
cles depending on duration of starvation. In parentheses are the total number of cells tested

Duration of starvation /hours/	1	2	3	12	24	Linear regression coefficient
Latex	9.32 (584)	11.01 (566)	11.72 (571)	6.31 (100)	1.40 (602)	-0.96
Carmine	4.39 (500	4.92 (500)	4.33 (400)	3.29 (100)	1.71 (500)	-0.98
Carmine + Albumin	8.33 (400)	7.58 (400)	6.19 (400)	4.47 (100)	3.59 (400)	-0.91
Carmine + Bacteria	6.28 (100)	5.60 (100)	–	–	1.11 (100)	-0.99

(Row label at left, rotated: Number of food vacuoles/cell)

As shown in Fig. 1, generally the rate of food vacuoles formation decreased
with the extension of starvation time, however, during the first 3 h of starvation
it remained almost at the same level. It is clear that the lowest rate of endocytosis
is noted after 24 h of starvation especially in cells which were fed with latex beads.
More than 60% of cells do not form vacuoles at all (Fig. 1). The high number of
paramecia without food vacuoles was found in cells treated with carmine particles
separately or with carmine particles with bacteria. The rate of endocytosis in paramecia
fed by carmine suspension mixed with bovine albumin decreased evidently after
24 h of starvation, though the percentage of cells not forming food vacuoles at
all did not exceed 5%.

The food vacuoles formation rate is different under the four types of experi-
mental conditions, if compared after the same time of starvation (Table 1). Since
the distribution of the number of food vacuoles formed is only approximately
normal, the mean values given in Table 1 have a tentative character. A comparison
of mean values enables us to draw a general conclusion that after a long-lasting
starvation the edible particles are taken up more willingly than inedible ones. In
the case of mixture of carmine with bacteria the rate of food vacuoles formation
is similar to that caused by inedible substances.

The linear regression coefficient of four examined types of particles demonstrated in Table 1 distinctly evidences a high correlation between starvation time and the number of food vacuoles formed in *Paramecium bursaria* cells.

Discussion

The rate of endocytosis is clearly connected with the number of particles in the medium (Mast 1974, Wichterman 1953, Chapman-Andresen and Nilsson 1968, Nilsson 1976, Wasik 1983), but as it was postulated by Nilsson (1972), even a few particles are able to initiate the food vacuoles formation process. Ricketts (1972) suggests that in *Tetrahymena* the rate of endocytosis is not an all-or-none phenomenon and that the endocytotic response is proportional to the degree of stimulation. In our previous (Wasik 1983, Wasik et al. 1984) studies it was confirmed for *Paramecium bursaria*. In the present experiments the number of particles remains constant (10^8 particles/ml), but we used two different kinds of suspensions: edible and inedible one. The different rate of food vacuoles formation is in that case connected with the nature of particles used.

The present observations have shown that the duration of starvation of *Paramecium bursaria* cells influences distinctly the rate of food vacuoles formation. The essential differences are clearly seen when the surrounding medium is replaced by mineral maintenance solution and ciliates are starved for 24 h prior to feeding (Fig. 1). As it was found the distinct role in this process plays the nature of particles supplied to be taken up as food. Carmine suspension and polystyrene latex beads used as indigestible material, were took in by *Paramecium bursaria* less readily than the same carmine particles, but mixed with bovine albumin (Table 1). Starved *Tetrahymena pyriformis* take up only edible material, while fed ones take in either useful or indigestible particles (Ricketts 1971 b, 1972). According to Seaman (1961), washed *Tetrahymena* cells do not take up the trypan blue, while if the dye is mixed with peptone-yeast it is readily taken up. Müller et al. (1965) demonstrated different results. His experiments showed that polystyrene latex particles (PLP) are ingested by *Paramecium multimicronucleatum* and *Tetrahymena pyriformis* irrespective whether the ciliates were fed or starved. Therefore Müller et al. (1965) concluded that there is no differences in the rate of uptake between nutritive and non-nutritive particles. Chapman-Andresen and Nilsson (1968) found that *Tetrahymena* exposed to a 53-hour period of starvation do not change the rate of food vacuoles formation in experiments with carmine suspension. The cessation of endocytosis was correlated only with the cell's stage in the cell life cycle (Chapmann-Andresen and Nilsson 1968, Nilsson 1976).

As it was suggested by Elliot and Clemmons (1966) starved *Tetrahymena pyriformis* can select the particles in the medium and prefers the edible ones. When the cells were fed on the suspension of gold, they formed food vacuoles, but if they

were fed with a mixture of gold and bacteria, only bacteria were taken up. On the other hand, *Tetrahymena* was not able to distinguish the bacteria if mixed with India ink. The food vacuoles containing both kind of particles were formed as readily as if exposed to the ink separately. *Paramecium bursaria* fed with the mixture of bacteria and carmine response generally as if exposed to carmine only, however, the rate of food vacuoles formation after short-lasting starvation is considerably higher in comparison to that obtained with carmine. Nevertheless these observations are consistent with Elliot and Clemmons (1966) and might suggest that the soluble factor (albumin) influence inedible matter (carmine) more distinctly than bacteria. It should be mentioned that *Klebsiella aerogenes* were washed in maintenance solution before use.

Our observations indicate that the rate of food vacuoles formation in *Paramecium bursaria* depends on the duration of starvation. It resembles the response of *Tetrahymena*. With the presence of inedible particles in the surrounding medium the percent of cells which do not form food vacuoles after a long-lasting starvation increases distinctly. In the case of edible particles the starved *Paramecium bursaria* behave like *Tetrahymena* (Ricketts 1971 b). The probable interpretation of this phenomenon was postulated by Ricketts (1971 b). When inedible particles were mixed with substances known as inducers of endocytosis (Seaman 1961, Chapman-Andresen and Nilsson 1968, Stockem and Wolfarth-Botterman 1969, Ricketts 1971 a), like protein, their surface changed. These substances alter the chemical or/and electrical nature of particles (Ricketts 1971 b).

As it was found previously (Wasik 1983, Wasik et al. 1984), different kinds of particles in the surrounding medium accelerate in *Paramecium bursaria* not only the food vacuole formation process, but the cytoplasmic streaming velocity too. The concentration of suspension was the factor influencing the intensity of both phenomena. When the medium was deprived of solid particles, the enhancement of endocytosis and cytoplasmic streaming flow did not occur (Wasik and Sikora 1984 a). This suggests the important role of particulate material in the surrounding medium, as a stimulating factor of both processes and supports the idea of the presence of receptors on the *Paramecium* body surface, postulated by Wyroba and Brutkowska (1978), Wyroba (1981) and Sikora and Jurand (1984).

Our previous (Wasik and Sikora 1984 b) and present observations lead to the suggestion that *Paramecium bursaria* may have different kinds of receptors on their body surface. They could be located on the surface or on the membrane of the cytostome. The presence of such receptors was postulated by Ricketts (1972) in *Tetrahymena pyriformis*, probably near the oral region of the cell or on the oral ciliature as bristles (Sattler and Staehelin 1974). Since the necessity of mechanical (Müller and Törö 1962, Müller et al. 1965, Wasik 1983, Wasik and Sikora 1984 a, b) or chemical (Seaman 1961, Seaman and Mancilla 1963, Ricketts 1971 a, 1972) triggering of endocytosis in ciliates was postulated, the presence of receptors for the regulation of uptake mechanism is highly

probable (Csaba 1985). The postulated receptors in starved cells had to act selectively, or the long-lasting starvation led to the blocking or disappearance of some of them. In such a condition there would be no signal to initiate the food vacuoles formation process. Some receptors remain functional and possibly are responsible for keeping the endocytosis on the low level. The hypothetical receptors responsible for the stimulation of endocytosis seem to be not Ca^{2+}-dependent. Kaczanowska (1978) found that *Paramecium tetraurelia* "pawn" mutant, which is deprived of the voltage-dependent Ca^{2+} excitability (Kung 1975) is not deprived of endocytotic activity and is able to form food vacuoles at the normal rate.

The results presented in this paper suggest the occurrence of receptors on the body surface of *Paramecium bursaria*, since the ciliate is able to select information about the nature of particles in the medium to be taken in.

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Determination of Cytosolic Sodium in *Stentor* Cells with Monensin-based Na+-Selective Microelectrode

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Synopsis. To measure cytosolic Na+ activities a double-barrelled Na+-selective microelectrode was constructed using monensin-based liquid ion-exchanger. The average values of slope constant and detection limit of ten tested microelectrodes were 58.6 mV and 0.01 mM of Na+, respectively. The selective coefficient for sodium over potassium was 13.4 and electrical resistance was in order of 10^{10} ohms with a response time less than 5 s. Using such type of microelectrode, the resting membrane electrical potential and intracellular sodium activity were measured in protozoan cell *Stentor coeruleus*. In cells with membrane potential more negative than -48 mV the cytosolic sodium ionic activity was determined as a value of 2.8 mM. It was concluded that fabricated monensin-based Na+-selective microelectrodes were suitable for intracellular application for protozoan cells and can provide valuable data on intracellular activities of sodium ions.

Cytoplasmic sodium ions in animal cells play an important role in a variety of cell functions such as generation of action potential and its transmission, muscle cell contractions, or can modulate the contractile vacuole activity in protozoan cell as well (Lee and Dagostino 1982, Allen and Navran 1984, Frixione and Perez-Olvera 1985). Changes in sodium ion concentration in the cell cytoplasm as well as changing in electrochemical gradient across cell membrane could affect the function of the cell in different ways. Ionic sodium is a direct regulator of the plasma membrane Na+-pump, which in turn influences the cell energetics (Kirschner 1964, Hoffman 1980). Changes in ionic activities of sodium could profoundly alter other intracellular ion concentrations. For example, discharge of H+ ions from the cell, with a concomitant rise in intracellular pH, could accompany Na+ influx via the Na+/H+-exchange system; or a change in intracellular Ca^{2+} may occur during Na+ influx via Na^+/Ca^{2+}-exchange mechanism (Parker 1978, Deitmer and Ellis 1980, Reeves and Sutko 1980, Grover et al. 1981, Boron and Bul-

paep 1983, Bridge and Bassinghwaighte 1983, Grinstein et al. 1984, Bayerdorfer et al. 1985, Ellis and MacLeod 1985). Intracellular pH, Ca^{2+} or Mg^{2+} ions may in turn be intimately involved in fine control of cellular functions. Further, changes in Na^+ activity in cytoplasm may highly modify contractile vacuole activity, a regulatory system of osmotic pressure and ionic composition of cell cytoplasm (Connoly and Kerkut 1983, Frixione and Perez-Olvera 1985). In particular, contractile vacuole activity has been associated with the coupled extrusion of sodium and water from protozoan cells.

It is therefore of considerable physiological interest to study the intracellular sodium ion activity or other ion activities and their transport across surface membrane in intact cells with ion-selective microelectrode technique.

Materials and Methods

Experiments were done on green ciliated protozoan cell *Stentor coeruleus*. Ciliates were cultured in semi-dark condition. at room temperature. Food source for the protozoans were axenic cultures of *Tetrahemyna pyriformis*. Stentor's culture medium included following salts: 10^{-3} M of NaCl, KCl, $CaCl_2$ and $MgCl_2$ and buffer solution of 10^{-3} M TRIS-HCl at pH 7.4. Cells chosen for the experiments were preincubated in fresh culture medium without the nutritional component for the period of 12 h and then were transferred into the experimental chamber.

The chamber had a volume of about 0.8 ml and was perfused at a rate of about 1.5 ml/min. Tested solutions during experiment could be changed without interrupting the fluid flow by means of a 6-way tap mounted near the chamber. All experiments were done at temperature of 17.5°C, controlled by Peltier device.

The double-barrelled ion-selective microelectrodes were fabricated as follows. Borosilicate glass capillaries (1B150F, W-P. Instr., USA) with 1.5 mm in outer diameter, 1.1 mm in inner diameter and 15 mm length were used. Two capillaries were mounted on a horizontal micropipette puller, gently rotated 180° along their longitudinal axes during the heating with a platinium ribbon, and drown carefully in two-step pull, i.e., initially pulled manually by approximately 1 mm and then pulled automatically by machine to a fine tip of 1 μm or less.

For membrane potential measurements, one of two barrels was filled with 10^{-1} M KCl from the stem end of the capillary. To determine the intracellular activity of sodium ions, the remaining barrel of the electrode was filled with sodium-selective liquid ion-exchanger. To perform this procedure successfully, the internal surface of the ionic barrel had to be made hydrophobic (at the very tip at 250–500 μm from the tip), while the remainder should be kept hydrophilic. The technique for siliconization in general has been described elsewhere (Walker 1971, Cole and Tsacopoulos 1977, Fabczak 1983). For the double-barrelled microelectrode, the following procedure should be carried out. First, after the tip of membrane potential sensing barrel had been completely filled with pure acetone from the stem end of the capillary, the tip assembly of two barrels was dipped into a silicone solution (TMSA, Serva) for several seconds to make the other tip of ion sensing barrel silicone filled. Second, the silicone- and acetone-holding double-barrelled micropipette was baked in the oven at 200–250°C for a period of 0.5–1 h to permit evaporation of the solvent. Then liquid ion-exchanger was introduced into the tip by a suction up to the desired height.

The ion-exchanger was a H^+-monensin (Sigma) dissolved in Corning resin (477317, Corning Co., USA, Kotera et al. 1979). The internal solution of ionic barrel contained 0.49 mM NaCl and 0.01 mM KCl. The pH of internal solution for sodium sensing barrel was adjusted to about 3 with 10^{-4} M citrate buffer to lower the pH sensitivity of the microelectrode.

Measurements of membrane potential difference and ionic electrochemical gradient were performed with a conventional and a high input impedance amplifiers, respectively (Fabczak 1983). Both amplifier outputs were connected to the digital voltmeters and displayed on a storage dual beam oscilloscope (5103N, Tektronix, USA).

Results and Discussion

The physico-chemical characteristics of ten tested monensin-based sodium-selective microelectrodes at room temperature were as follows. At sodium concentrations higher than 10^{-3} M changes in electromotive forces of the electrode (EMF) were linear at the slope of 56.8 mV per 10-fold sodium concentration change (Fig. 1).
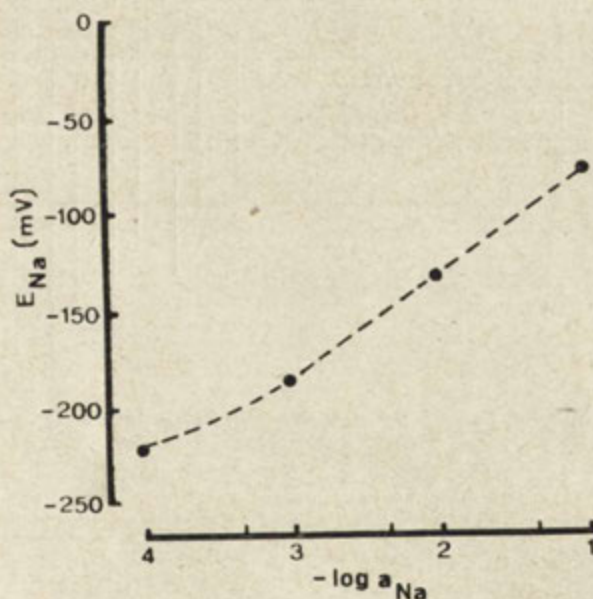

Fig. 1. Voltage (EMF) response calibration curve for monensin-based sodium-selective microelectrode across range of NaCl concentrations (i.e., 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M) at a constant ionic strength ($i = 0.1$) adjusted with KCl solution

Electrode detection limit was around 10^{-5} M of Na^+. The electrode selectivity constants for sodium over potassium, estimated by the separate solution method (Oehme et al. 1976) following UIPAC recommendation (Guilboult et al. 1978), were 14.3, while ion interferences of Ca^{2+}, Mg^{+2} and H^+ were negligible. Figure 2 illustrates an experiment in which resting membrane potential (A) and cytoplasmic sodium (B) were measured in *Stentor* cell over a period of more than half an hour. A double-barrelled Na^+-selective microelectrode (i.e., membrane potential and ionic barrels) was first kept outside the cell for some time to equilibrate. Then the cell was penetrated with the electrode in the frontal region of the cell body by means

of mechanical micromanipulator. As the cell was punctured, the membrane potential recorded by the conventional barrel was automatically substracted from the EMF of the Na^+-selective barrel (Fig. 2 from the moment marked by arrow). The
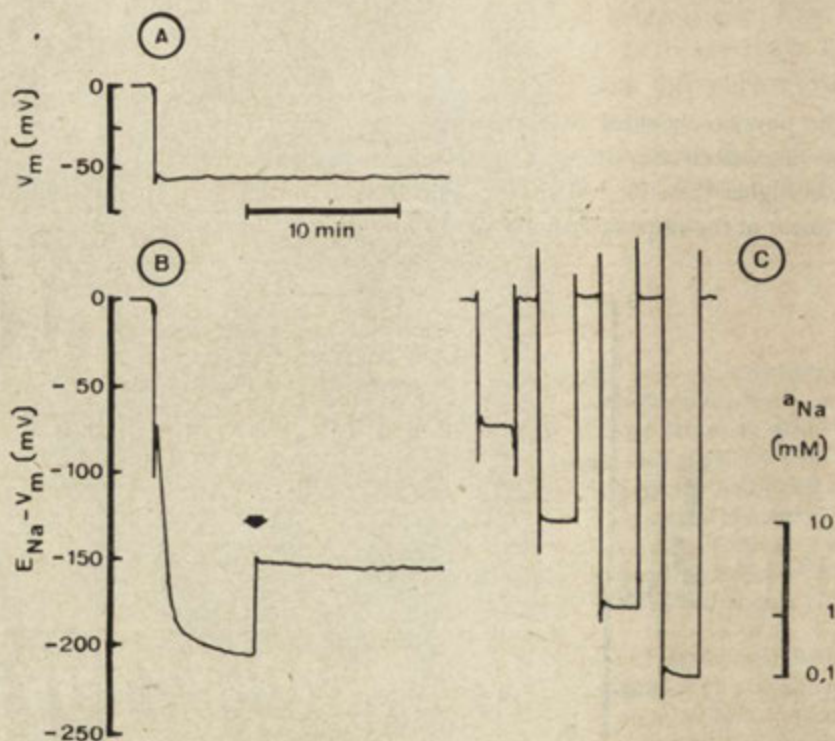

Fig. 2. Membrane potential (A) and cytoplasmic sodium (B) of *Stentor* cell. After the withdrawal of the sodium-selective microelectrode from the cell it was calibrated (C) by perfussing the experimental chamber with NaCl solutions of 0.1, 1, 10 and 100 mM. Arrow marks the onset of electronic membrane potential substraction from voltage of ionic electrode response (EMF)

correct cell penetration with the microelectrode was continuously monitored by current passing through the cell membrane and measuring the resulting changes in the membrane potential. The membrane potential recorded in the cell was close to -51.5 mV throughout the experiment (Fig. 2). The cytoplasmic magnitude of sodium ion activity was about 3.6 mM after penetration and cell body contraction, falling later to about 2.8 mM over the next 10–15 min. Such a change in internal sodium activity from the value observed in the first minute after penetration was found in almost all the cells being tested and was presumably due to recovery of the cell body from contracted (excited) state and recovery from membrane damage as well.

Determinations of cytoplasmic ionic sodium were therefore made only when the internal values of Na^+ had recovered from the initial rise. The intracellular

sodium activity measured in several cells of *Stentor* was 2.8 ± 0.2 mM (mean value of 18 measurements $\pm$ S.D.). The corresponding value from membrane potential was equal to -47.8 ± 1.6 mV. In almost all the tested cells with a resting membrane potential below the value of -48 mV there was a significant correlation between the magnitude of membrane potential and the intracellular sodium activity. This may be somehow related to both the activity of Na^+/K^+-exchange system and the osmotic equilibrium of water across the cell membrane (Connoly and Kerkut 1982).

Apart from some brief observations with a single-barrelled microelectrode (Fabczak 1986) the only other measurement of internal sodium concentration in *Stentor* cells is that reported by Wood (1973) who used an ultramicroflame photometry system. The value of cytoplasmic sodium concentration of 0.1 mM reported by Wood is over 20 times lower than that found in the present experiments. It is difficult to find the cause of such discrepancy. It might depend on different condition of cell cultures or physiological solutions tested. Moreover, as it was pointed out above, the intracellular sodium ion activity (concentration) can vary with membrane potential fluctuation or with the level of internal potassium activity (concentration). Thus, the internal sodium levels obtained in different experiments cannot be compared unless the data on simultaneous membrane or potassium electrochemical potential are presented.

Data in the present paper distinguishly indicate that the monensin-based sodium-selective microelectrode can be exploited to measure the ionic sodium in protozoan cells *in vivo* the more so as the level of ion can vary with changes of cell physiological state, sometimes without a change in total ion content. Such ion-seective microelectrode study is leading to a better understanding of the cytosolic behaviour of sodium or other ion as well, within an intact and not only protozoan lcells.

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The Effects of Tetraethylammonium on the Excitability of Marine Ciliate *Fabrea salina*

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Synopsis. Externally applied TEA evoked periodic ciliary reversal (PCR) in *Fabrea salina*. In the presence of TEA in the medium the following data on *Fabrea salina* were obtained: (a) the resting membrane potential was depolarized (by 50% in 100 mM TEA) and the membrane input resistance was increased (by 40% in 100 mM TEA), (b) the overshoot of a potential response to injected current was increased and the prolonged depolarization occurred, (c) the action potential exhibited two peaks: graded one and "all-or-none" one, (d) the amplitude of barium spikes was increased by a factor of 2.5 (in 25 mM TEA) and their frequency was much lower, (e) K+-induced ciliary reversal was prolonged. It is suggested that Ca^{2+} (Ba^{2+}) conductance, undetectable in the absence of TEA, may exist in the *Fabrea* membrane. The differences in excitable membrane properties between *Fabrea* and fresh water ciliates are discussed.

The marine heterotrich ciliate *Fabrea salina* is grown in highly concentrated salt solution (artificial sea water concentrated by a factor 2.6). Similarly as in fresh water ciliates — the excitability of marine ciliates is Ca-dependent (Deitmer and Machemer 1982 — *Paramecium calkinsi*, Kubalski 1983 — *Fabrea salina*). In contrast to the "all-or-none" Na spikes in metazoan cells, the action potentials exhibited by ciliates are graded according to the strength of stimuli. "All-or-none" nature of the regenerative calcium activation is balanced by potassium activation. Tetraethylammonium (TEA+) is known as ion blocking both the voltage dependent K conductance (e.g., in the frog myelinated nerve fibre — Hille 1967) and the resting K permeability (e.g., in the barnacle muscle — Keynes et al. 1973). In the presence of TEA the Ca activation is more effective and therefore it is more easily detectable. Regenerative calcium dependent responses are sometimes not seen, unless the delayed rectification is reduced with TEA (Katz and Miledi 1969 — the frog neuromuscular junction). There were several studies on the effects

of TEA on the excitability of fresh water ciliates (see, for example Friedman and Eckert 1973, Satow and Kung 1976) — the present study reports some findings obtained on *Fabrea salina* when its potassium conductance is suppressed or nearly abolished.

Material and Methods

The *Fabrea salina* strain (obtained from the Laboratory of Zoology, Ecole Normale Superieure, Paris) was grown in the medium: 1100 mM NaCl + 130 mM MgCl₂ + 70 mM Na₂SO₄ + 25 mM CaCl₂ + 22 mM KCl + 5 mM NaHCO₃ + 5 mM Tris/HCl (pH 7.2) with an addition of *Aerobacter aerogenes* as standard food supply. Cells used for experimentation were kept in unbacterized medium for 1–2 days. The standard control solution was: 1100 mM NaCl + 130 mM MgCl₂ + +25 mM CaCl₂ + 22 mM KCl + 5 mM Tris/HCl (pH 7.2).

In all experimental solutions the ionic strength was kept constant. When the concentration of KCl was increased or when BaCl₂ and/or TEA (from Merck, West Germany) were introduced into the medium, the NaCl concentration was decreased by the osmotically equivalent amount.

Direct observations of behaviour were done under low magnification of an optical microscope.

The method used for intracellular recordings has been described elsewhere (Kubalski 1983). Some data mentioned in Section I of "Results" were obtained in the Lehrstuhl für Allgemeine Zoologie at the Ruhr-University in Bochum on the set-up described by de Peyer and Machemer 1977). The microelectrodes were filled with 3 M KCl.

Results

I. Effect of Externally Applied TEA on Action Potentials

When 50 mM TEA is present in the experimental solution, the cells respond with a few minutes of periodic ciliary reversal (PCR). The reaction is very similar to that shown in Ba solution called "barium dance" (Dryl et al. 1982). Each transient period of ciliary reversal is accompanied by an "all-or-none" action potential.

A similar effect of TEA was also demonstrated by Satow and Kung (1976) in the fresh water ciliate *Paramecium aurelia* in the presence of 4 mM TEA. The PCR behaviour is temporary, after a few minutes the transient periods of ciliary reversal appear less frequently until they decay. The threshold concentration for inducing the PCR response is about 30 mM TEA, in higher concentration (75 mM) a short-lasting immobilization of the movement is followed by the PCR response with slightly prolonged periods of backward swimming.

When the effect of TEA was examined under voltage-clamp conditions 50 mM TEA reduced the amplitude of the maximum outward current by a factor of 2–3, whereas a TEA concentration of 100 mM reduced the outward current 3–5 times (Kubalski and Machemer-Römisch — unpublished). Thus, for the purpose of investigating the K-channel blocking effect of TEA, action potentials were recorded in the presence of 100 mM TEA. In this TEA concentration the resting mem-

brane is depolarized by up to 50% and the membrane input resistance increases by 40%. In these conditions *Fabrea salina* cells respond to injected currents with the action potentials graded in the amplitude and the rate of rise. Figure 1 shows three responses to injected currents (2.5, 5, 10×10^{-9}A, 50 ms) in the absence (Fig. 1 A and Fig. 1 A^1) and in the presence (Fig. 1 B and Fig. 1 B^1) of TEA.

Figures 1 A and 1 A^1 (also Fig. 1 B and Fig. 1 B^1) represent the same traces recorded at a different time base. In the TEA solution all three responses evoked by
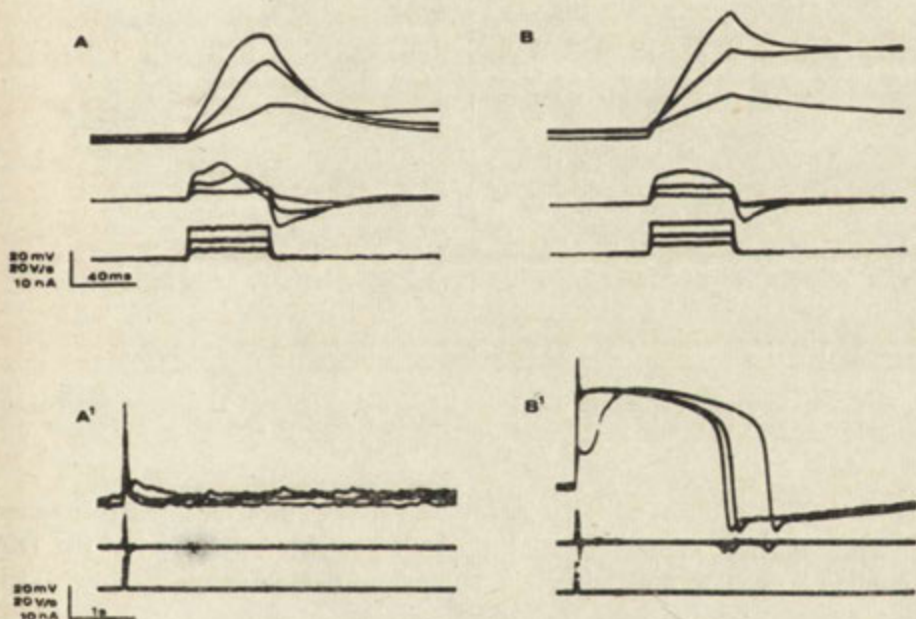

Fig. 1. The effect of externally applied 100 mM TEA on potential responses to injected currents. A, A^1 — action potentials in standard solution, B, B^1 — after 3 min of exposure to TEA. The time base in A and B is 40 ms/div, in A^1 and B^1 — 1s/div. The three traces in each frame represent: potential response, first derivative of the potential changes dV/dt and injected currents 2.5, 5, 10×10^{-9} A from top respectively. Note the prolongation of the spikes after applying TEA and the "all-or-none" character of the prolonged depolarization

injected outward currents reach higher peaks and their maximal dV/dt traces (middle traces in Fig. 1) are all smaller than those in the control medium. The action potentials are significantly prolonged in the presence of TEA (Fig. 1 B^1), which is in agreement with data demonstrated in *Paramecium* (Naitoh et al. 1972). The action potentials following mechanical stimulation in *Stylonychia* were also prolonged after application of TEA (Deitmer 1982). The down stroke of the action potential is only influenced by TEA in fresh water ciliates — unlike in *Fabrea*. *Fabrea salina* in the TEA solution exhibits two-peak electrically induced action potentials — the first graded according to the injected current and the second "all-or-none" in character, with a steady state depolarization lasting up to several seconds in response to stronger depolarizing outward currents (Fig. 1 B^1).
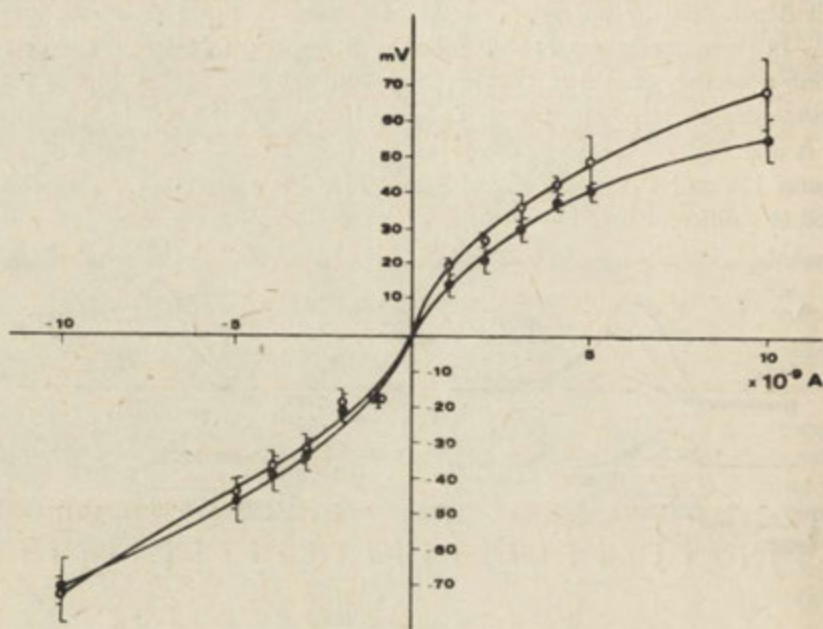
Fig. 2. I–V relationships of *Fabrea* in standard solution and in 100 mM TEA solution. Filled circles and bars are means ± S.D. (*n* = 5–14) in the standard medium, open circles and bars (*n* = 7) in the presence of TEA

Current-voltage relationships at steady-state were plotted for standard medium and in the presence of 100 mM TEA (Fig. 2). The significant effect of the TEA application is observed in the depolarization half of the I–V curve.

II. Effect of Externally Applied TEA on "All-or-none" Action Potentials in Ba Solution

Kinosita et al. (1964) demonstrated that paramecia responded with "all-or-none" spikes when exposed to 2 mM $BaCl_2$ (in the presence of 1 mM $CaCl_2$). Further studies on *Paramecium caudatum* showed that Ba^{2+} could carry the transmembrane current efficiently and block the K leakage — which resulted in "all-or-none" electrogenesis (Naitoh and Eckert 1968). "All-or-none" action potentials were also recorded in *Paramecium aurelia* (Kung and Eckert 1972, Satow et al. 1974), *Stylonychia mytilus* (de Peyer 1973) and *Fabrea salina* (Dryl et al. 1982, Kubalski 1983).

The response of *Fabrea salina* to 50 mM $BaCl_2$ (25 mM $CaCl_2$ solution) in the absence and in the presence of 25 mM TEA is shown in Fig. 3. Addition of TEA is marked by an arrow. Within the first 3 min after TEA has been applied, the am-
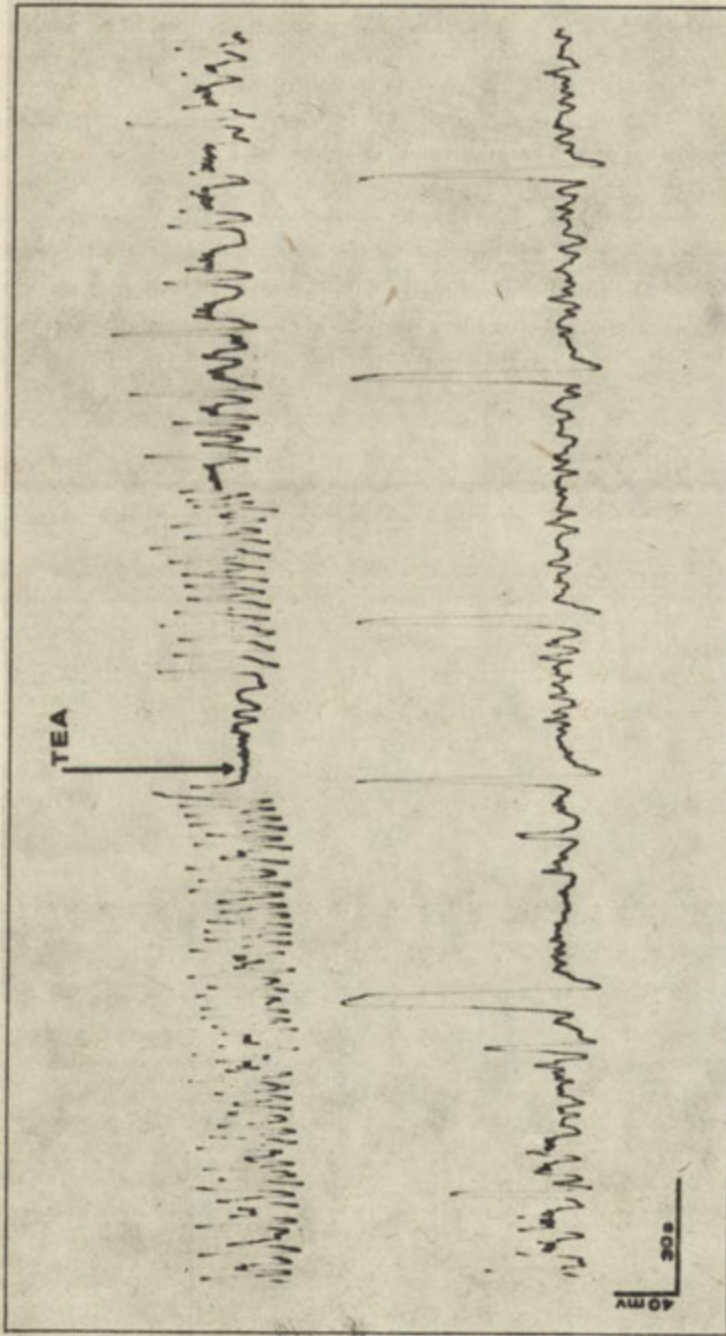
Fig. 3. Ba-induced "all-or-none" action potentials before and after applying 25 mM TEA. Arrow "TEA" indicates the time when the TEA solution begins to be introduced to the experimental chamber. Note the changes in amplitude and frequency of the Ba-spikes

plitude of some spikes is unaffected (60–65 mV); some spikes exhibit higher amplitude (up to 90 mV) and sometimes the prolonged depolarizations (up to 20 s) occur.

In the solution without TEA, *Fabrea salina* exposed to $[Ba^{2+}]/[Ca^{2+}]$ ratio = 2/1 exhibits action potentials lasting up to 5 s (Fig. 3, Kubalski 1983). After some delay, a dramatic change in the amplitude and in the frequency of generated action potentials can be observed. The amplitude is increased by a factor of 2.5 and the frequency of action potentials is approximately 1 spike per 1 min. Those strong "all-or-none" action potentials can be recorded for 5–10 min. Later on, they become more frequent and their amplitude return to its initial value. Increasing the concentration of $BaCl_2$ (in the presence of 25 mM $CaCl_2$) prolongs the time duration of action potentials both in the absence and in the presence of 25 mM TEA, while the amplitude of the spikes is increased by approximately the same factor.

III. TEA Prolongs K⁺-induced Ciliary Reversal

The threshold concentration of KCl for inducing a continuous ciliary reversal (CCR) in *Fabrea salina* is 60 mM (in the presence of 25 mM $CaCl_2$). As it was reported for *Paramecium*, higher concentrations of ammonium ions salts (chloride and acetate) prolonged K⁺-induced ciliary reversal (Doughty 1978). Alkyl derivatives of quaternary ammonium ion salts TEA and TMA prolong the duration of K⁺-induced CCR response when applied at low concentrations, but at higher concentrations they induce the ciliary reversal themselves (Doughty and Dryl 1981, review). Upon the transfer to TEA solutions, *Fabrea salina* adopts a PCR response — i.e., the intermittent reversed swimming behaviour very similar to that shown in Ba solution (see Section I in this paper). The threshold concentration of TEA for inducing PCR response is about 30 mM thus two lower concentrations (10 and 20 mM) were used to investigate its effect on K⁺-induced ciliary reversal. Ten and 20 mM TEA depolarize the resting membrane by up to 20 and 25% respectively.

Table 1

The duration of ciliary reversal response induced by K⁺ in standard medium and in the presence of 10 and 20 mM TEA

Concentration of KCl (mM)	Duration of CR response		
	in standard medium (in min)	in standard medium +10 mM TEA (in min)	in standard medium +20 mM TEA (in min)
50	lack of CCR	1.5±0.1	15.0±6.1
100	6.5±3.0	12.0±5.3	45.0±12.3
150	40.0±13.0	42.5±9.1	<90.0

All responses to different K^+ concentrations in both 10 and 20 mM TEA are longer (see Table 1) than in the control solution. The results mentioned in Table 1 represent the total time duration of reversed swimming behaviour i.e., the time until 50% of specimens show a recovery of the forward movement. It is worth to note, that in the presence of TEA the duration of CCR is slightly prolonged and a marked increase of duration is observed in the partial ciliary reversal sequence (PaCR, Grębecki 1965) i.e., spinning, circling and looping until the cell readopts the forward left spiraling movement.

Discussion

Externally applied TEA, although not so effective as injected intracellularly, slows down the repolarizing phase of the action potential in *Paramecium caudatum* (Naitoh et al. 1972) and enhances the amplitude of the regenerative calcium response (Friedman and Eckert 1973). These authors showed also that internally applied TEA increases membrane resistance and blocks the downstroke of the Ca response. Satow and Kung (1976) demonstrated similar findings on *Paramecium aurelia* and showed that the amplitude of the action potentials in the solution containing 4 mM TEA was graded with respect to the applied stimulus up to approaching an "all-or-none" responses — further increase in outward current strength did not alter the peak potential but increases the rate of rise. It was also shown in *Paramecium aurelia* that internally applied TEA reduced the resting potential and the rate of rise of the Ca action potential. All these apply also to *Fabrea salina*. Externally applied TEA reduces the resting membrane potential and increases its input resistance. There is, however, one difference: i.e., *Fabrea* in the TEA solution responds to the injected currents with prolonged depolarization — unlike *Paramecium*. In *Paramecium aurelia* a decrease in $[Ca]_{in}$ (realized by EGTA injection) is a prerequisite for the prolonged depolarization. There are two components in the potential response of *Fabrea* in TEA solution. The first peak is graded with the stimulus and its amplitude is enhanced comparing with the corresponding one in the control solution. It was shown in barnacle muscle fibers (Hagiwara et al. 1964) that the peak potential of Ca-spike is determined by both Ca and K currents. The outward K current may overlap the Ca inward current even at the time when it shows its maximum. Therefore, the overshoot of the action potential increases with an increase of the Ca current and/or decrease of the K current (Hagiwara et al. 1974). In *Paramecium* neither the extracellular TEA (Satow and Kung 1979) nor the ionophoric injection of TEA (Brehm and Eckert 1978) affects the amplitude or kinetics of early inward current. Thus, the enhancement of the peak potential in the presence of TEA can be only caused by K-channel blocking properties of TEA. The second component of the action potential of *Fabrea* in the TEA solution is the prolonged depolarization with an "all-or-none" character. The prolongation of the action potential after the suppression of K conductance may indicate the lack

of Ca-inactivation or the presence of a late Ca current. Both possibilities may occur — the lack of Ca-inactivation was demonstrated in barnacle muscle fibers (Keynes et al. 1973) and the presence of a late Ca-current was shown in a Helix neuron (Meech and Standen 1975). Two-peak action potential under normal conditions was demonstrated in the fresh-water ciliate *Stylonychia mytilus* with the graded and the "all-or-none" components (de Peyer and Machemer 1977). After a further voltage-clamp analysis of transmembrane currents in *Stylonychia* it was concluded, that the two components of the action potential may correspond to the two different Ca conductances (Deitmer 1984).

Progressively longer sustained depolarization is also observed while increasing external Ba concentration. Ba ions suppress the K current and the degree of this suppression increases with increasing Ba concentration (Hagiwara et al. 1974). A delay in the activation of repolarizing mechanisms of the Ba induced action potential is a result of the suppression or absence of the K outward current. On the other hand, Ba ions are able to carry more current through the Ca channel, independently of any effect on the K channel (Hagiwara et al. 1964, 1974).

Fabrea salina exposed to Ba solution in the presence of TEA exhibits "all-or-none" action potentials with prolonged depolarization shortly after the application of TEA. Later on, the prolonged depolarization is not observed but the amplitude of the spikes rises 2–3 times. This strong effect of TEA on Ba action potentials might be explained by additive blocking K conductance properties of TEA — on the one hand, but on the other — this finding may reflect also the presence of $Ca^{2+}(Ba^{2+})$ conductance, which can be only seen and recorded when the K outward rectification is suppressed. Two-peak potential responses to injected currents recorded in the presence of TEA may also support this suggestion.

The prolongation of the ciliary reversal response induced by K^+ in the presence of 10 and 20 mM TEA may indicate another physiological effect of TEA on the properties of the cell membrane of *Fabrea*. The presence of externally applied TEA depolarizes the cell membrane (20 mM TEA by up to 25%), thus the application of lower concentration of K^+ may evoke the same ciliary response as higher concentration in the absence of TEA — if we take into consideration only the depolarizing effect of TEA. Higher concentrations of TEA evoke a periodic ciliary reversal response as a consequence of reducing the short-circuiting effect, what results in the appearance of spontaneous activity.

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Analysis of Cytochemical Procedure of Precipitation of Calcium Ions by N,N-naphthaloylhydroxylamine (NHA) in *Acanthamoeba* Cells

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Synopsis. The process of precipitation of calcium ions by a Ca-trapping agent, N,N-naphthaloylhydroxylamine, is examined. The optimal conditions for the precipitation of calcium ions in test tubes as well as in *Acanthamoeba* cells are described. Special attention is focused on the retention of cellular calcium during preparation of the cells for electron microscopy examination. Localization of cellular calcium in *Acanthamoeba* cells is presented.

Many attemps have been made to localize calcium at cellular and subcellular levels in different biological material. For this purpose cytochemical, autoradiographical and X-ray microanalysis techniques combined with electron microscopy have been used (Caswell 1979). Localization of calcium in cells and tissues is usually studied by cytochemical techniques in which calcium ions are precipitated by chelators and form the insoluble electron-dense deposits detectable under electron microscope. The most popular Ca^{2+}-capture agents are oxalate and pyroantymonate anions (Podolsky et al. 1970, Coleman and Terepka 1972, Wick and Hepler 1982). Oxalate has high selectivity but low sensitivity for calcium (Caswell 1979). Pyroantymonate forms insoluble electron-dense products also with the sodium and magnesium ions (Komnick 1962, Torack and LaValle 1970, Klein et al. 1972). However, several modifications of pyroantymonate technique have been developed which, according to their authors (Slocum and Roux 1982, Wick and Hepler 1982, Borgers et al. 1984) allow to precipitate only calcium ions within the cells.

Besides these two compounds the cellular calcium may be also precipitated by N,N-naphthaloylhydroxylamine. This Ca-trapping agent was primary used for quantitative determination of calcium content in serum (Beck 1951). Later the reagent was applied in cytochemistry for visualization of cellular calcium

under light (Voigt 1957) and electron (Zechmaister 1979, Przełęcka et al. 1986) microscope.

The Zechmaister's procedure seems to be insufficient for complete precipitation of all the original cellular calcium. In this procedure the cytochemical reaction for visualization of cellular calcium was performed in primary fixed and washed tissues. However, it is well known that during fixation of biological material without a calcium-trapping agent the main fraction of cellular calcium is lost (Van Iren et al. 1979). Therefore in our study we examine the process of precipitation of calcium ions by NHA and describe the optimal conditions at which the precipitation and retention of precipitated calcium occur in test tubes as well as in *Acanthamoeba* cells. The visualization of cellular calcium in *Acanthamoeba* is also performed.

Material and Methods

Culture. *Acanthamoeba castellanii* (Neff strain) was grown in proteose peptone-yeast extract, the optimal growth medium, as it was described previously (Sobota et al. 1984).

Precipitation of calcium ions in test tubes. N,N-naphthaloylhydroxylamine sodium salt (Fluka) was dissolved in 3-fold glass-distilled water as 50 mM stock solution before each of 3 to 6 h experiments. The precipitation of calcium ions by NHA was carried out in plastic test tubes during 2 h at room temperature in solution containing 2% glutaraldehyde, 100 mM collidine buffer, pH 7.4, and radioactively labelled calcium ($^{45}CaCl_2$) in concentration indicated at given experiments. The concentration of calcium-capture agent NHA was 5 mM. The total volume of the incubation medium was 1.0 ml. The precipitates were collected by centrifugation (11,000 g, 10 min, 4°C). Samples of supernatants were dried in scintillation vials and, after an addition of liquid scintillator, counted in Beckman liquid scintillation counter.

In some series of experiments the formed precipitates were washed and treated similarly as it was done during preparation of the samples for electron microscopy. Namely, the formed Ca-NHA precipitates were washed twice in solution containing 50 mM NaCl, 100 mM collidine buffer, pH 7.4, with or without 5 mM NHA. The washed precipitates were treated with 1% osmium tetroxide containing the same concentrations of NaCl, collidine and NHA as in washing solution. After the treatment the precipitates were collected by centrifugation as above, washed again and dehydrated with the graded ethanol (30, 70, 100%). The 200 μl samples of supernatants were counted in liquid scintillation counter.

Retention of cellular calcium during fixation of *Acanthamoeba* cells in the presence of NHA. The cells loaded with $^{45}Ca^{2+}$ were washed twice with solution containing 120 mM NaCl and 20 mM Hepes buffer, pH 7.4. During the third washing the solution contained also 0.5 mM LaCl₃. Lanthanum ions being a good calcium analogue remove the externally bound calcium ions (Langer and Frank 1972). Subsequently, the cells were washed twice with NaCl-Hepes buffer solution and fixed with 2% glutaraldehyde containing 120 mM NaCl, 50 mM collidine buffer, pH 7.4, with or without 5 mM NHA. One hour later the fixed samples were rinsed three times with NaCl-collidine washing solution with or without 5 mM NHA. The samples were dehydrated with a series of ethanols (30, 70 and 100%). At each step the supernatant was collected and its 0.5 ml sample was dried in scintillator vial and counted in a liquid scintillation counter.

Preparation of *Acanthamoeba* cells for electron microscopy. *Acanthamoeba* cells were fixed in 2.5% glutaraldehyde solution containing 120 mM NaCl, 50 mM collidine buffer, pH 7.4, and 5 mM NHA as calcium-capture agent. Two hours later the cells were briefly washed twice with

a solution: 120 mM NaCl, 50 mM collidine buffer, pH 7.4, and 5 mM NHA. The samples were postfixed with 1% osmium tetroxide containing NaCl-collidine-NHA, and one hour later the samples were again washed twice with NaCl-collidine-NHA solution. Dehydration of the samples was performed with the graded ethanol (30, 70, 100%) to which 5 mM NHA was also added. The control samples were prepared without NHA in all the used solutions. Epon-embedded material was cut on a LKB ultratome and examined with an electron microscope JEM 100B.

Results and Discussion

Figure 1 shows the extent of precipitations of calcium ions depending on concentration of NHA in incubation medium. Progressively increasing concentration of NHA removed calcium ions from incubation medium. The complete precipitation of Ca^{2+} was observed at above 3 mM NHA. On the basis of the above data 5 mM NHA was used as Ca^{2+}-trapping anion for further experiments.
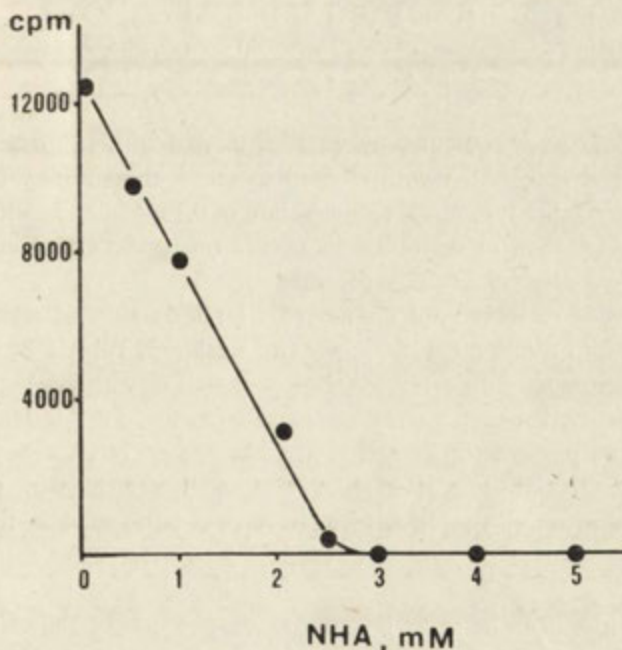

Fig. 1. Effect of different concentrations of NHA on the precipitation of 0.1 mM ⁴⁵Ca-labelled CaCl₂. Extent of calcium precipitation was evaluated by residual radioactivity of ⁴⁵Ca²⁺ (cpm) in supernatants

Stability of Ca-NHA Precipitates. Intending to use the Ca-NHA precipitates for localization and visualization of cellular calcium ions under electron microscope, the Ca-NHA precipitates formed in test tubes were further treated in a similar manner as it was done during preparation of biological samples for electron microscopic examination. Therefore, Ca-NHA precipitates labelled with

Table 1

Losses of $^{45}Ca^{2+}$ from ^{45}Ca-labelled Ca–NHA precipitates during their treatment
with solutions containing or lacking 5 mM NHA

Treatments	^{45}Ca-radioactivity detected in supernatants			
	Solutions with NHA		Solutions without NHA	
	A		B	
	cpm	%	cpm	%
Total radioactivity of Ca–NHA precipitates	159,929	100	167,482	100
First washing	1130	0.7	4483	2.7
Second washing	950	0.6	1746	1.0
1% osmium solution	860	0.5	1680	1.0
Washing	670	0.4	1630	1.0
30% ethanol	560	0.3	1520	0.9
70% ethanol	185	0.1	375	0.2
100% ethanol	138	0.1	483	0.3
Total	4493	2.7	11,917	7.1

radioactive $^{45}Ca^{2+}$ were subsequently washed, treated with 1% osmium tetroxide,
washed again and dehydrated with ethanol to check the stability of the precipi-
tate. As shown in Table 1, in result of incubation of 0.1 mM $CaCl_2$ with 5 mM NHA
a complete precipitation of calcium ions occurs (insignificant amounts of $^{45}Ca^{2+}$
radioactivity were detected in the supernatants).

The washing of the precipitates removed a large fraction of calcium ions from
the precipitates into solution (ca. 3.7% per two washings, Table 1 B). Further treat-
ment of the precipitates with osmium solution as well as with rinsing solutions and
ethanol also removed a certain fraction of calcium (ca. 3.4%). The total loss of
calcium from the precipitates treated in the absence of NHA amounted 7.1%.

However, if the similar procedure of washing, treatment with osmium tetro-
xide, and dehydration of Ca–NHA precipitates was performed in the presence of
5 mM NHA, no significant amounts of calcium ions were detected in supernatants
(Table 1 A).

To study a loss of cellular calcium during preparation of the cells for electron
microscopic examination, *Acanthamoeba* cells were loaded with $^{45}Ca^{2+}$. After the
radioactive calcium ions were supplied into growth medium, an accumulation of
radioactive calcium by the cells was detected (2873 cpm per 10^6 cells of 7-days
old culture). Fixation of *Acanthamoeba* cells with glutaraldehyde supplemented
with 5 mM NHA caused practically complete precipitation of cellular calcium
which was pelleted together with the fixed cells (traces of radioactivity were detect-
ed in the supernatant).

The treatment of fixed cells with the solutions lacking in NHA evoked a release
of a certain part of precipitated calcium, which thus appeared in supernatants

(ca. 25%). Subsequent washing and dehydration of the postfixed cells also resulted in appearance of radioactivity in supernatants though a significantly smaller one (ca. 7%) than that observed after the first two rinses (Table 2 B). However, if the solutions used for washing and dehydration were supplied with 5 mM NHA, no

Table 2

Losses of ^{45}Ca^{2+} from glutaraldehyde-NHA fixed *Acanthamoeba* cells during preparation of the cells for electron microscopic examination. The cells were treated with solutions containing or lacking 5 mM NHA

Treatments	^{45}Ca-radioactivity detected in supernatants			
	Solutions with NHA		Solutions without NHA	
	A		B	
	cpm	%	cpm	%
Total ^{45}Ca-radioactivity of glutaraldehyde-NHA fixed cells	26,006	100	26,393	100
First washing	652	2.5	2685	10.0
Second washing	293	1.1	3946	15.0
Third washing	371	1.4	1319	4.9
30% ethanol	183	0.7	475	1.8
70% ethanol	291	1.1	54	0.2
100% ethanol	226	0.8	120	0.5
Total	2016	7.6	8599	32.4

significant amounts of calcium ions were detected in the corresponding supernatants (Table 2 A). The results indicate that if the entire procedure of fixation and washing of the cells was performed in the presence of 5 mM NHA, the initially precipitated cellular calcium stayed inside the cells as unsoluble Ca-NHA complexes. Therefore, for visualization of cellular calcium 5 mM NHA should be present at all the steps during preparation of the cells for electron microscopic examination.

Plate I shows an *Acanthamoeba* cell, which was prepared for electron microscopic examination in the presence of 5 mM NHA. In result of this procedure the electron-dense material in cytoplasm, mitochondria, lipid droplets as well as at the plasma membrane may be distinguished. Some precipitates were also observed outside the cells on the outer surface of plasma membrane. The electron-dense material and high electron-density of plasma membrane are supposed to reflect the localization of cellular calcium, which interacts with NHA and gives electron-dense microprecipitates.

The presented data confirm the ability of NHA to precipitate calcium ions completely from the solutions in test tubes as well as in *Acanthamoeba* cells. The most serious problem of the used method is proved to be a dislocation and loss of calcium ions, particularly during processing of the cells for electron microsco-

pic examination. Fixation of the cells and tissues in the absence of calcium-capture agent evokes a lost of ca. 80–90% of all the cellular calcium (Van Iren et al. 1979). However, if the preparation of cells for electron microscopy is performed in the presence of antimonate (Ca-trapping anions) only 25% of cellular calcium is lost (Van Iren et al. 1979). Substantially less loss of cellular calcium has been reported by Slocum and Roux (1982), who propose a modified pyroantymonate procedure of precipitation of cellular calcium, which allows to retain ca. 90% of cellular calcium. In our experiments the use of NHA as a Ca-capture agent allows to retain ca. 93% of cellular calcium under an indispensable condition of carrying out the whole procedure (i.e., the preparation of the cells for electron microscopic examination) in the presence of NHA. Such a condition makes the method suitable for visualization and localization of almost all calcium in cells and tissues.

ACKNOWLEDGEMENTS

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PLATE I
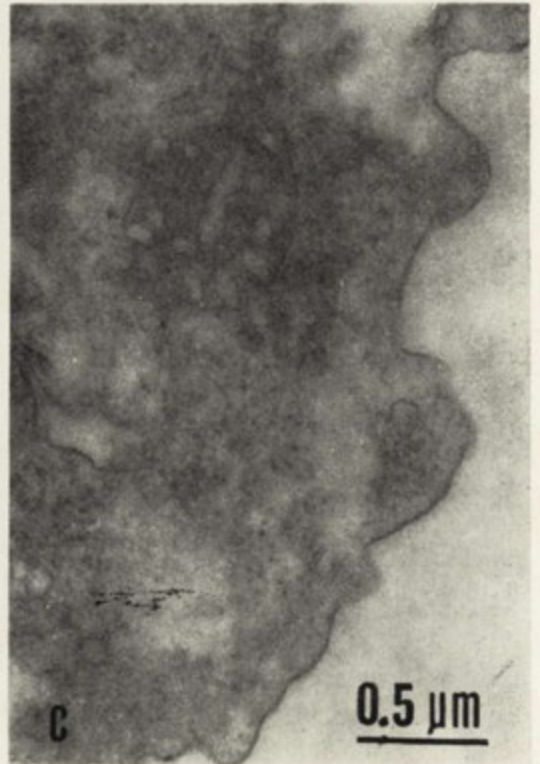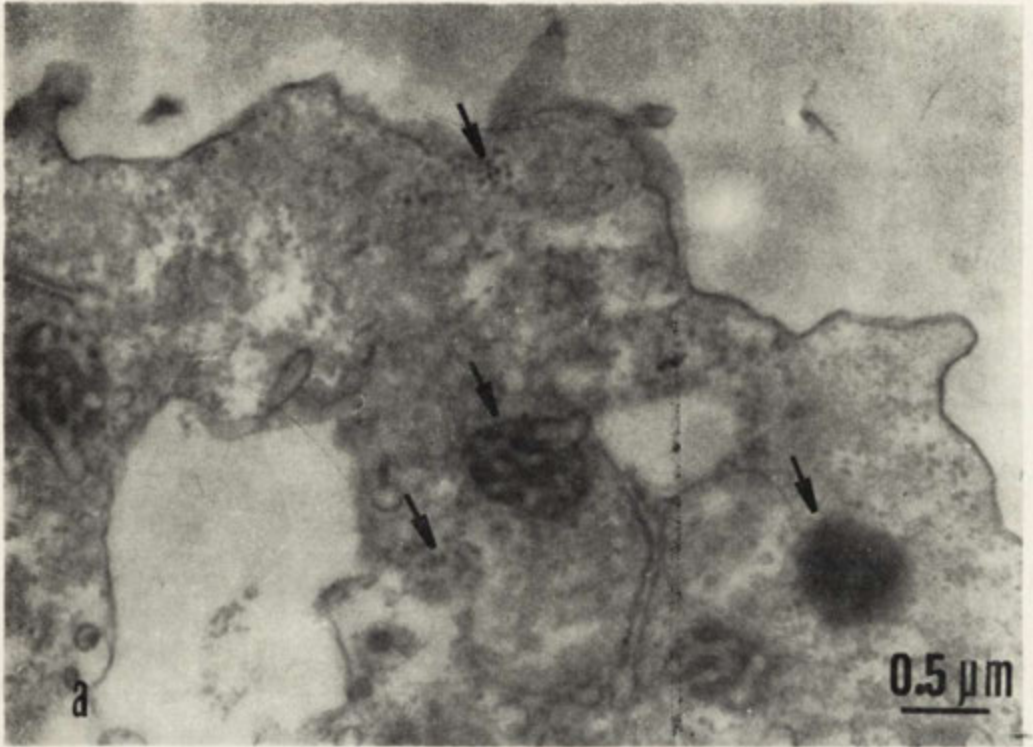
Cytochemistry and Life Cycle of *Trypanosoma cruzi* Chagas, 1909

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Synopsis. The review contains the result of cytochemical research of DNA, RNA, nucleotides, proteins, thiol groups, phosphorus, iron, potassium, NAD-H and NADP-H-tetrazolium reductases, cytochrome oxidase, peroxidase, polysaccharides in the complex life cycle of protozoan Chagas disease agent *Trypanosoma cruzi*. On the base of these data the metabolic nature of *T. cruzi* conversions during its life cycle, its adaptation to various environmental conditions, the pre-adaptation to life cycle continuation under other conditions and the influence of the parasite on human cells are considered. The cytochemistry of the *T. cruzi* cell as such, its nucleus, flagellar apparatus, chondriome and kinetoplast are analysed.

For many years the cytochemical study of the Chagas disease agent has been carried out at the Moscow University headed by professor Roskin. These investigations covered all the stages of the *Trypanosoma cruzi* development both in culture (promastigote, epimastigote and metacyclic forms) and in vertebrate host — mice (amastigote forms in tissues and trypomastigote ones — in blood), as well as in tissue cultures (Fig. 1). They have been performed by using a large number of cytochemical reactions, characterizing different aspects of cell metabolism. As a result of this investigation *T. cruzi* appeared to be the best cytochemically studied species, at least among *Trypanosomatidae*.

The concrete results of the studies of RNA (Kallinikova and Roskin 1963 a, Khlgatyan 1965 a), DNA, proteins, histones (Kallinikova and Wartoń 1972), nucleotides (Balicheva and Roskin 1963), polysaccharides (Kallinikova 1964), thiol groups (Roskin and Kozukhova 1964), oxidative enzymes (Khlgatyan 1965 b, Kallinikova 1967 a, 1968 a, b), potassium as well as iron and phosphorus bound with organic compounds (Roskin and Kolomina 1964), together with some of the summaries (Kallinikova and Roskin 1963 b, Roskin and Kallinikova 1963, Roskin et al. 1968, Kallinikova 1967 b, 1969 a, b) are published mostly in Russian. That is the only reason why these investigations are not sufficiently known to foreign scientists. Nevertheless, they substantially add to the general picture of *T. cruzi* cell biology observed in the last review (De Souza 1984).

Fig. 1. Life cycle and RNA of *Trypanosoma cruzi*. Stages of the life cycle: P — promastigote, E — epimastigote, M — metacyclic, forms, A — amastigote, T — trypomastigote. Cellular structures: n — nucleus, fl — flagellum, um — undulating membrane, bb — basal body, k — kinetoplast

The purpose of the present review is to sum up in brief the investigations of Roskin's group and to consider them from the view point of cellular differentiation, life cycle and trypanosomatids ontogenesis.

These works have shown on the whole that in the course of the complex life cycle *T. cruzi* cell, along with well known morphological changes (motor apparatus first of all), undergoes very consistent cytochemical alterations.

The well known metabolic changes in the trypanosomes life cycle, that is the qualitative alterations of respiratory metabolism in the African species, is not valid for *T. cruzi*. According to some biochemical data (Brand and Johnson 1947, Baernstein and Tobie 1951, Baernstein 1953, Seaman 1953, Agosin and Brand 1955, Brand and Agosin 1955, Baernstein 1963) this species possesses the cytochrome system and Krebs cycle at all stages of its development. Having applied cytochemical methods, we and other authors (Chakravarty et al. 1962, Pizzi 1950) observed mitochondrial activity (particularly succinate-dehydroge-

nase and terminal oxidase) not only in epimastigote and trypomastigote forms covered by biochemical studies, but at all stages of the *T. cruzi* life cycle. Basing on cytochemistry, a terminal oxidase is the cytochrome oxidase and the variation of its activity at different steps of *T. cruzi* development is insignificant. At the same time the pre-cytochrome oxidase enzyme system is not quite the same at different stages of the cycle. Diaphorases (NAD-H- and NADP-H-tetrazolium reductases), which have not been studied well enough in *Trypanosomatidae*, are found cytochemically at all the stages as well, but their activity is not uniform and is especially slight in metacyclic and trypomastigote forms. Trypomastigotes possess only NADP-H-diaphorase. The peroxidase connected with non-cytochrome oxidation has been found by us only at the epimastigote stage. Thus, contrary to Boveris et al. (1980) at least at this cycle step *T. cruzi* is defended against H_2O_2 and free radicals connected with H_2O_2. *T. cruzi* is potentially capable of doing without the cytochrome system, although it posesses the latter. It was possible to reveal peroxidase activity in trypomastigote *T. cruzi* as well by electron-cytochemical methods (Wartoń and Modlińska 1977, De Souza 1984).

Thus, cytochemical investigations show that though *T. cruzi* does not undergo such dramatic changes of its oxidative metabolism as African trypanosomes do, the oxidative abilities of this species, despite the apparent uniformity (De Souza 1984) do not prove to be similar at different steps of the life cycle.

Besides oxidative enzymes, the content, topography, revealing form of RNA, proteins, free purine ribonucleotides, polysaccharides, thiol groups, potassium, as well as phosphorus and iron associated with organic substances also alter in the course of *T. cruzi* development. As a result, each stage of the cycle has its own cytochemical characteristics.

As a whole, the development stages of *T. cruzi* in culture (similar to those in an invertebrate host) are more complicated and richer in the studied substances and the activity of the oxidative enzymes as compared with the stages in a vertebrate host. It corresponds to the results of the mitochondria studies under the light (Pizzi 1950) and electron microscopes (Sanabria 1963, 1964) and to the concept of a greater metabolic activity of *T. cruzi* in an invertebrate host (Hutner and Lwoff 1951, Brand 1960), which have been based on biochemical data.

Among the cultivated forms promastigotes are especially active in reactions for RNA, nucleotides and organic phosphorus, while epimastigotes are richer in polysaccharides and thiol groups. For the karyosome of both stages intensive reactions for RNA, nucleotides, phosphorus, thiol, proteins are very specific. It runs in line with the other authors' impressions (Esponda et al. 1982) of the metabolic activity of this epimastigote organelle. Another peculiarity of both cultivated forms is their cytochemical polarization, that is the concentration of RNA, proteins, nucleotides, oxidative enzymes (and polysaccharides in the logarithmic phase of growth) in the anterior part of the cell, especially around the kinetoplast and the base of the flagellum.

In culture only metacyclic forms (Fig. 1) are remarkable for the poverty and dispersion of RNA (which corresponds to the poverty of endoplasmic reticulum — Brack 1968) as well as extremely low protein, nucleotides, thiol groups content and a slight activity of diaphorases. They are also remarkable for the decrease of cytochemical polarization. On the pale background of the cell only the flagellum, kinetoplast and adjacent cytoplasmic zone demonstrate intensive reactions.

In a vertebrate host intracellular *T. cruzi* amastigote forms are characterized by two cytochemical properties: (1) the extreme decrease and even complete absence of some reactions (for potassium and SH-proteins), (2) the localization of many substances, like proteins, polysaccharides, oxidative enzymes along the border of the host cell. It corresponds to the data on weak basophilia of amastigotes (Wood 1953), the poverty of endoplasmic reticulum (Brack 1968, Meyer 1968) as well as with the electronograms (Sanabria 1964) showing the concentration of their mitochondria in the periphery of the cytoplasm.

According to the same data (Wotton 1940, de Meirelles and De Souza 1982), *T. cruzi* trypomastigotes prevailing in the blood at the peak of infection differ from amastigotes in a higher mitochondrial activity. The results of our work show that they are characterized by a higher cytochrome oxidase activity, large quantity of phosphorus, SS-proteins and especially of granular RNA, the content of which is comparable with that in epimastigotes and even in promastigotes. This runs in line with the abundant cytoplasmic granulae (Ormerod 1958) and the highly expressed endoplasmic reticulum (Brack 1968) in trypomastigotes. However, unlike pro- and epimastigotes and despite the richness in RNA, trypomastigotes are as poor in proteins and nucleotides as amastigotes. Slight protein synthesis in those forms has been shown by biochemical methods as well (Pizzi and Taliaferro 1960). As in metacyclic forms, the activity of diaphorases in trypomastigotes is very low, cytochemical polarization is smoothed and it is only in the flagellum and kinetoplast that the reactions for proteins, nucleotides, thiol groups and polysaccharides are intensive. The area between nucleus and kinetoplast corresponding to Golgi zone is free from RNA and is posititive in reaction for polysaccharides. The cellular membrane of trypomastigotes contains polysaccharides as well. But while in the flagellum and kinetoplast they are represented by glycogen, in the cell membrane and Golgi apparatus they are represented by glycoproteins.

As we have shown recently (Kallinikova 1982, Kallinikova and Donchenko 1985), pleomorphism (tetramorphism) is characteristic of the trypomastigote stage of *T. cruzi* life cycle. It suggests the existence of C-slender, C-middle, S-slender, and S-middle forms as well as the regular change of these morphological types in the infection development. Everything mentioned above refers only to the middle (mainly C-middle) forms, which represent the majority at the peak of parasithemia and are usually considered in literature. Slender trypomastigotes being typical for the beginning of the infection and not numerous enough, differ from the middle forms in poor RNA content and its dispersive form and seem to

be richer in proteins. Janus green B is positive only in their kinetoplast. The Golgi apparatus is less developed in them (Brener 1973). The contradiction between our data and the literature ones (de Meirelles and De Souza 1982) in respect to the diaphorases activity of *T. cruzi* at this stage, can be explained by its pleomorphism and unequal enzyme activity of different trypomastigote forms.

The cytochemical pecularities of the observed stages reveal the metabolic nature of *T. cruzi* transformations in its life cycle.

The transformation of promastigotes into epimastigotes is not only a translocation of kinetoplast, the flagellum base in the direction of the nucleus and the appearance of undulating membrane sprouts, but also a certain impoverishment, of the cell in RNA (and the change of its boulder form into a granular one), nucleotides, organic phosphorus, as well as the escape of SH-containing proteins from karyosome. Simultaneously polysaccharides and thiol group content increase and change of their composition, the nucleus is being enriched in iron and the cell acquires peroxidase activity.

Much more substantial are the alterations during the transformation of epimastigotes into metacyclic stage. While the motor apparatus becomes more and more complicated and the active reactions to energetically important substances (polysaccharides, nucleotides, phosphorus) in it and in the kinetoplast are being conserved, there occurs a sharp impoverishment of the whole cell in RNA, proteins, nucleotides and thiol groups and impoverishment of the nucleus and kinetoplast in iron. Peroxidase activity disappears, cytochrome oxidase and diaphorases activity decreases, mitochondrial activity appears at the pole of the cell opposite to the kinetoplast, the polysaccharides composition changes.

The transformation of very mobile metacyclic forms into intracellular amastigotes lacking the free flagellum and undulating membrane is not accompanied by serious cytochemical changes in spite of dramatic morphological and physiological alterations. The picture of RNA remains the same, the cell membrane is being strongly enriched in polysaccharides and proteins, the polysaccharides composition changes, the nucleotides, phosphorus and potassium contents continue to fall and they almost disappear, while diaphorases activity somewhat increases, thiol groups appear in the nucleus and iron is detectable in the nucleus and kinetoplast.

On the whole, *T. cruzi* at its trypomastigote stage is not very active in most reactions. During the transition of intracellular amastigotes into trypomastigotes, mainly into middle C-forms, a part of cytochemical reactions does not change (SH-groups of free cysteine, polysaccharides), while others become weak (total and SH-proteins, diaphorases, iron). However, this transformation is accompanied by the change of polysaccharides composition (the substitution of easily dissolved glycogen for glycoproteins in the flagellum and kinetoplast) and mainly by the sharp increase in RNA, phosphorus and partly nucleotides content as well as cytochrome oxidase activity. Appearing earlier than the middle trypomastigotes, the slender ones are intermediate between the middle forms and the amastigotes. They

have not yet undergone the alterations mentioned above and seem to smooth out the differences between these forms.

The transformation of *T. cruzi* blood forms into promastigotes in the culture means a sharp activization of cell metabolism and a substantial increase in the majority of cytochemical reactions. Granular RNA turns into the boulder form, iron again appears in the nucleus and kinetoplast, the changes in SS-groups and cytochrome oxidase reactions are not so significant. On this background the motor apparatus is not so distinguished as in trypomastigotes.

Thus, cytochemistry shows that metabolic changes from stage to stage of *T. cruzi* life cycle are not less significant than in African tryponosomes and they concern various aspects of cell metabolism.

In cytochemical alterations of *T. cruzi* are reflected, first of all, physiological properties of the parasite at different steps of the cycle and the adaptation to various environmental conditions.

Low intensity of most cytochemical reactions distinguishing trypo- and amastigotes from the ones in culture or in invertebrate host is certainly connected with their deeper and specialized parasitism and greater dependence on the host, the host cell. The concentration of most substances at the periphery of the amastigote cytoplasm indicates a close interaction between the parasite and host cell cytoplasm. The localization of mitochondria (Sanabria 1963, 1964), respiratory enzymes (de Meirelles and De Souza 1982) and glycogene (Kallinikova and Danilova 1968) of the host cell around amastigotes can demonstrate the same fact. The concentration of parasite acid mucopolysaccharides on this border makes it possible to assume the participation of the system hyaluronic acid-hyaluronidase in the mechanism of *T. cruzi* intracellular invasion.

We have used cytochemical methods to study not only the parasite itself, but its influence on human cells in tissue culture as well (Kallinikova and Danilova 1968).

The first cytochemical changes in cells of HeLa and HEp-2 cell lines occur before the penetration of parasites into them. They precede morphological alterations, touch upon energy, carbohydrates and lipids metabolism and result in a fast and considerable depression of mitotic activity. The penetration of parasites and their multiplication within the cell provoke its morphological changes (hypertrophy, vacuolization, distortion in the nucleus), alterations in the nucleic acids-protein metabolism, further decrease of respiratory enzyme activity and the mitosis number. Complete degeneration and cell death occur only when most of the cells become infected and when amastigotes start to retransform into trypomastigotes.

The fact that Chagas disease agent passes through several stages of development in human cells and makes the maximal use of them shows that the given parasite-host system is very well correlated and evolutionary steady. This relationship seems to be less sharp than that of *Leishmania donovani* and *Leishmania major* with the same tissue cultures. The advantage of *T. cruzi* amastigotes in the interac-

tion with a vertebrate cell is expressed cytochemically by the fact that even if they are not transformed into trypomastigotes and die together with the cell, their cytochemistry remains normal for a longer period of time.

At the moment of maximal invasion some changes occur in uninfected cells as well. Pathogenesis of Chagas disease does not appear to concern only the intracellular invasion itself, though *T. cruzi* differs from many other trypanosomes by the presence of intracellular stage of development. The parasite is likely to excrete some toxins, this corresponds to certain clinical data.

It must be noted that four *T. cruzi* strains avirulent for animals, kindly donated by Dr. Packchanian (USA), were not able to develop in HeLa and HEp-2 tissue cultures. Perhaps tissue cultures may be a simple and fast method for the determination of *T. cruzi* strains virulence. And artificial conditions of such development are not an obstacle to this purpose. Despite this artificiallity, the ability of *T. cruzi* virulent strains to develop in human cancer cells, which served as the basis of cancer biotherapy (Kluyeva and Roskin 1946, 1963) is realized in tissue cultures.

The cytochemical peculiarities of amastigotes show the lack of some autonomous syntheses at this stage of *T. cruzi* development. Only cytochrome oxidase and diaphorases activity remains high enough. But this stage is a temporary step which is followed by stages with active and autonomous syntheses. Some of them are already reconstructed at the next, trypomastigote stage.

The cytochemical character of this stage reflects its presence in the liquid medium and especially in blood, where immunological interaction of the parasite and the vertebrate host takes place. High activity of the flagellar apparatus in many cytochemical reactions on the one hand and certain evidence of Golgi apparatus activity on the other hand are connected with the conditions mentioned above. The high content of RNA in trypomastigotes in the second half of the infection course, the presence of glycoproteins in Golgi zone and the cell membrane can indicate the intensive synthesis of antigens in these forms. By analogy with African trypanosomes, it can be supposed that they are glycoprotein antigens which are concentrated in Golgi zone and evacuated onto the cell surface in the form of the so-called coat or endogenous glycocalix. Similar carbohydrate-protein complexes in connection with the cell membrane were discovered by us and other authors (De Souza and Meyer 1975) in amastigotes, that is at the stage where immunological interaction with a vertebrate host also occurs. At both stages other intracellular carbohydrates are lacking, as it was observed before (Lillie 1947, Schulz and Mac Clure 1961).

The intensification of *T. cruzi* cytochemical reactions while returning to the culture from the vertebrate host indicates greater metabolic activity in these conditions and a smaller dependence on the host during the development in the invertebrate host.

The cytochemical characteristic of a stage also reflects the preadaptation to the

transition into the next stage and to the cycle prolongation in other conditions.

First of all preadaptation is expressed in the properties of the stages which are responsible for the transfer to the other host. The cytochemical peculiarities of metacyclic forms which distinguish them from other stages of development in culture or in the invertebrate host: impoverishment of the cell in essential substances and the decrease of oxidative enzymes activity — is the approach to the properties of following amastigote stage, i.e., adaptation which enables the development in the vertebrate.

Trypomastigote forms carry out the return to the invertebrate host. Preadaptation to this moment of the life cycle is morphologically expressed by the appearance of wider (middle) forms which, unlike slender ones (Silva 1959), are capable of living and developing in the bug. We have shown that preadaptation at this stage has its cytochemical expression as well. Slender trypomastigotes emerging at the beginning of infection and unable to develop in the bug are similar to the previous stages (metacyclic and partly amastigotes) in many cytochemical properties, first of all in RNA. At the same time middle trypomastigotes, characteristic for the peak of infection and responsible for the prolongation of the life cycle in the invertebrate host, have common cytochemical properties with the next promastigote stage (Fig. 1).

The transition of amastigotes into slender trypomastigotes in the vertebrate host, despite its grave morphological changes, is not so substantial in a number of cytochemical patterns as the transition of slender trypomastigotes into middle ones. All this leads to the fact that many cytochemical changes of *T. cruzi* are most essential in two points of the life cycle connected with the change of host: (1) during the transformation of epimastigotes into metacyclic forms infective for a vertebrate and (2) at the trypomastigote stage while returning to an invertebrate. Consequently, cytochemical changes forestall the change of the host and divide the life cycle in another way than the host change itself. *T. cruzi* life cycle in its RNA cytochemistry is divided into two parts by the diameter passing between epimastigote and metacyclic stages in its invertebrate half and between slender and middle trypomastigotes in the vertebrate one (Fig. 1).

Perhaps the preadaptation to the life cycle prolongation is also expressed in the cytochemical heterogeneity of cell populations at each stage of *T. cruzi* development. This refers mostly to the kinetoplast, possibly to its ultrastructure (Meyer-1968) and chondriome as a whole (Paulin 1983) and can't be explained by division phases. That can indicate the heterogeneity of the population in certain properties, particularly in the readiness for transition into the next stage of the life cycle, which was observed during cloning. Epimastigotes of the same strain are not uniform in their ability to the transformation into metacyclic forms (Goldberg and Pereira 1981, Crane and Dvorak 1982, Domingues et al. 1982).

Cytochemical preadaptation to the life cycle prolongation is revealed in the development of each stage as well, that is in the succession of cell generations separa-

ting one stage from another and preparing the above mentioned metabolic and morphological transformations. Thus, gradual cytochemical changes of epimastigotes in the culture (within one passage), occurring on the background of active cell division in the subsequent morphologically identical generations and making them cytochemically different, precede the transformation into metacyclic forms. These changes are not only gradual, but also regular. RNA, nucleotides, organic phosphorus and SH-groups content in epimastigotes have two peak points: at the end of the logarithmic and at the end of the stationary growth phases. A somewhat different dynamics is characteristic for polysaccharides, peroxidase and proteins, including SS-binding ones. Their maximum is observed at the beginning of the stationary phase and the diffusion reaction is transformed into granular one. It is connected with the appearance of special cytoplasmic granulae of complex composition. In the course of culture growth cytochrome oxidase activity is progressively falling, while potassium and iron content remains unchanged. This agrees with biochemical data on the changes of respiratory potential in epimastigotes during the culture growth (Brand et al. 1946, Warren 1960, Zeledon 1960, Carneiro and Caldas 1982) as well as on the decrease of some syntheses and nucleic acids content during the transition into the stationary phase (Fernandes 1965, Fernandes and Castellani 1966).

Cytochemical data have shown that an increase in pH and cell number in old *T. cruzi* cultures (Chang and Nagherbon 1947, Chang 1948) represents a real animation. It is accompanied by partial normalization of epimastigotes' cytochemical characteristics and the appearance of new metabolic potentials: the ability of doing without peroxidase and of being satisfied with extremely low cytochrome oxidase activity.

The dynamic of cytochemical and biochemical changes shows that the competence to transformation into metacyclic forms arises (at the beginning of the stationary phase) at the dramatic moment of the culture growth. Epimastigotes are deformed, some of their cytochemical reactions undergo qualitative changes and temporary specific granulae and oxidative potentials appear. When transformation ability appears, the cytochemical properties of epimastigotes become preadaptive, because the impoverishment of the cell in the most essential compounds and the decrease of the oxidative enzymes activity are the very changes, which epimastigotes have to undergo in the course of transformation into the metacyclic stage.

The ability to only subsequent cell differentiation, the gradual character of cytochemical changes as well as the gradual acquirement of the competence to the transformation into the next stage cause a specific phenomenon. *T. cruzi* life cycle, similarly to some other parasitic *Protozoa*, is carried out by a succession of cell generations, which makes the ontogenesis of these species quite peculiar (Kallinikova and Severtzov 1983). Being ontogenesis of full value such life cycles are not limited by individual development only. They consist of series of genuine individual ontogeneses of the cells-specimens, the life of which is equal to the cell cycle

(from the mother cell division to the daughter cell division) and neither reproduced all the properties of the species nor represents its ontogenesis. However, cytochemical investigations showed that the role of such ontogeneses-cell cycles is so important, as it is owing to them that the differentiation of the cell as an organism as well as the reproduction of the species-specific complete life cycle becomes possible.

In such form of ontogenesis a certain rupture takes place between the individual development itself (the cell of a definite stage of a life cycle being the bearer) and the complete reproduction of species properties (the full life cycle being the bearer). This widenes the whole concept of the nature and evolution of ontogenesis as a general biological phenomenon. Evidently the essence of ontogenesis should be seen mainly in the reproduction of species properties, which may take place both in the populations of organisms (at least of unicellular ones) and in the course of individual development of the specimen (as it is accustomed). The complex life cycles of parasitic *Protozoa* like those mentioned above connect, in the evolutionary sense, the ontogenesis of many unicellular organisms equal to a cell cycle with the individual development of multicellular organisms, which again have a specimen as a bearer of species properties (at the new level of organization, with full subordination of the cell to the whole organism).

The given research of *T. cruzi* has one more aspect — the cytochemistry of the *Kinetoplastida* cell itself.

In accordance with the literature data (Lillie 1947, Pizzi 1950, Noble et al. 1953, Pizzi and Diaz 1954, Diaz 1955, Noble 1955, Schulz and Mac Clure 1961) we have observed Feulgen-positive material either evenly distributed in the *T. cruzi* nucleus, or in the form of a peripheral ring, or in the form of chromosome-like granulae, the number of which is difficult with accuracy to define. These differences rather reflect the steps of cell division. However, trypomastigotes unable to divide were also different in nucleus Feulgen-reaction, which can be explained by their polymorphism. For the widest forms, the fragmentation of chromatine material on chromosome-like granulae is specific.

Though the cytochemical and ultrastructural (Brack 1968) changes from stage to stage of *T. cruzi* life cycle less concern the nucleus, its composition does not remain the same. Beside DNA, the nucleus contains SS-groups and iron at all steps of the cycle except trypomastigotes. It may contain polysaccharides at the metacyclic stage, while at the trypomastigote stage (the only stage that was investigated in this respect) histones have been observed.   —

A positive correlation takes place between the contents of RNA in the cytoplasm and in the karyosome. A large quantity of RNA was discovered in the karyosome of actively dividing and RNA rich epi- and trypomastigotes. The karyosome has a small quantity of RNA in metacyclic and amastigote forms, which are poor in RNA. Trypomastigotes (middle ones), with RNA rich cytoplasm and relatively RNA poor karyosome are the only exception. *T. cruzi* karyosome also

contains free ribonucleotides (excluding metacyclic forms), organic phosphorus (in epi- and promastigotes) and SH-proteins (in promastigotes).

In *T. cruzi* cytoplasm special attention must be paid to the discovered specific granulae — temporary structures at the epimastigote stage. By cytochemical methods, they have been found to contain proteins, glycoproteins, thiol groups, peroxidase activity and the ability to stain with Janus green B. Probably these granulae contain glutathion-peroxidase, which was looked for but was not found by biochemists (Boveris et al. 1980).

The distinctive peculiarity of the *Trypanosomatidae* cell is the motor flagellar apparatus. While in all cytochemical reactions the undulating membrane looks very pale (but not hollow), the flagellum and its basal body are distinguished on the cell background by a high content of many substances, especially energy-supplying, and at the most mobile stages. The basal body contains RNA, nucleotides, polysaccharides, phosphorus, thiol groups, potassium at many stages of the life cycle.

The other peculiarity of *Kinetoplastida*, their united mitochondrial system (single megachondriosome) with a specialized part — the kinetoplast, is also likely to be connected with the presence of the flagellar motor apparatus. *T. cruzi* megachondriosome does not look like a united structure in any cytochemical reactions. Even the activity of respiratory enzymes has been observed as a discrete mitochondria only at some points of this formation. However, within this united chondriome, ats "nucleoid" (kinetoplast) shows cytochemical specificty, particulary in the comiosition of its surrounding membrane, which proves to be closed in all reactions pnd makes the kinetoplast a discrete independent organelle at the level of light microscopy.

Our investigations made it possible to discover a complex chemical composition of *T. cruzi* kinetoplast. It contains DNA, RNA, nucleotides, proteins, poly saccharides, phosphorus, thiol groups, iron, potassium and oxidative enzymes This allows the kinetoplast to react both as a nucleus and as a mitochondria.

T. cruzi is the first species where kinetoplast histones have been discovered. We have found histones in the kinetoplast of the trypomastigote forms. The previous contradictions concerning epimastigotes (Heroin-Delaeney 1965, Steinert 1965) can be considerably resolved by the new cytochemical (Ogloblina, personal communication) and biochemical (Saucier et al. 1981) data on the high protein content in the kinetoplast in general, and in connection with the kinetoplast, DNA in particular, only at the phase of active multiplication of these cells. The dependence of the histone content and topography in the kinetoplast on the stage of *T. cruzi* life cycle has been shown by the electron cytochemical methods. In metacyclic forms the histones are abundant and they are connected with the kinetoplast DNA fibrils. The histones are few in the kinetoplast of 3-days' culture epimastigotes and they are located at the periphery, outside the kinetoplast DNA Souto-Padron and De Souza 1978, Esponda et al. 1982).

As far as cytochemistry is concerned, *T. cruzi* kinetoplast proved to be a highly differentiated organelle, having dual chemoarchitectonics, which consists in a regular distribution of DNA and other compounds in the kinetoplast. Many of its components are localized in the achromatic part. The differences between the stages of *T. cruzi* life cycle mostly concern the kinetoplast, not only its morphology, but its cytochemistry as well. The kinetoplast's cytochemical characteristics also vary from specimen to specimen in the population at the same stage of the life cycle.

The above properties of *T. cruzi* kinetoplast proved later to be common for the majority of *Kinetoplastida* (Kallinikova 1977, 1981).

On the whole, the *T. cruzi* cell is highly differentiated in the cytochemical respect. It can be said that its cytochemical specificity is the concentration of the most important biochemical components near the basis of the flagellum and especially around the kinetoplast. The degree of such cytochemical polarization is connected with the structure of the flagellar apparatus. The polarization is greater in the forms where the base of the flagellum is located at the anterior pole of the cell (pro- and epimastigotes). At the stages which have no free flagellum (amastigotes) or where the flagellum together with the undulating membrane extends along the whole cell (metacyclic and trypomastigote forms), the biochemical components are distributed in the cell more uniformly.

More particular cytochemical properties of the *T. cruzi* cell are also connected with the degree of the flagellar apparatus development. The cellular RNA content decreases and its dispersion increases with the complication of this apparatus in the culture forms. The promastigotes possessing the flagellum only are rich in RNA in its boulder form. More motile epimastigotes having the flagellum and the undulating membrane embryo are less rich in RNA, RNA having a granular form. Metacyclic forms with their most perfect motor apparatus and extreme mobility are characterized by the lowest content of RNA and by RNA disperse form. In this succession the cell nucleotides and phosphorus content decreases. Thus, the principal specificity of the cells in question — the flagellar apparatus — leaves a distinct mark on their cytochemistry.

SUMMARY

In the course of the complex life cycle of the Chagas disease agent, qualitative and quantitative changes occur concerning different sides of the cell metabolism including oxidative one.

In the cytochemistry of stages their adaptation to various environmental conditions is reflected. The culture forms are richer, more active and more many-sided than the stages which develop in a vertebrate host. Among the latter the amastigotes are distinguished by the extreme weakening of most cytochemical reactions and by the concentration of the remaining reactions at the border of the host cell. Pleomorphism and cytochemical prominence of Golgi zone and motor apparatus are characteristics of trypomastigotes.

Preadaptation to the cycle prolongation is also reflected in *T. cruzi* cytochemistry. Cytochemical changes precede the change of the host. In the succession of cell generations separating one

stage from another, cytochemical changes are gradual and preadaptive. The cell population at any stage is not cytochemically homogeneous.

The Chagas disease pathogenesis does not come to intracellular invasion only. Cytochemical changes of human cells arise before the penetration of *T. cruzi* into them. These changes precede the morphological ones, they relate to the energy, carbohydrates and lipids metabolism and result in a rapid decrease of mitotic activity.

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Acanthamoeba rhysodes (Singh, 1952) (*Protozoa*: *Gymnamoebia*) from Gangetic-estuary, India. I. Population Growth Kinetics in Simplified Media

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Synopsis. Acanthamoeba rhysodes (Singh, 1952) strains (referred to as strains I, II and III for premonsoon, monsoon and postmonsoonal ones respectively) were isolated from the lowest low tide belt areas of Sagar Island (21°30' to 21°50' N and 88°04' to 88°08' E) facing Bay of Bengal. They were cultured in saline distilled water agar (SDWA) media of different strength with *Escherichia coli* as the food. The capability of the media of use to support growth of the organisms, morphometric measurements including locomotive rate and the overall growth after 100 days of culture were compared. It was evident that the investigated strains could grow well in all the media. Further, that they exhibited little variation in growth over changes of media salinity.

Amoebae may be found in almost any damp place — moist soil and sand, on aquatic vegetation, on wet rocks, in lakes, ponds, streams, glacial melt water, tide pools, bays, estuaries, on the bottom of or afloat in the open ocean, or on (or in) littoral organisms (Bovee and Sawyer 1979). These two authors (1979) add that the wide distribution of these organisms in coastal and estuarine benthic habitats is owing to wave action and ocean currents. It has been recognized that soil constitutes a reservoir for these amoebae to disseminate in other environments (Singh 1973).

As the maintanance of a continuous culture for amoebae is a prerequisite for further studies, different media are available in literature (Dixon 1937, Singh 1945, 1955, Prescott and James 1955, Prescott 1956, Kudo 1957, Chang 1958, Adams 1959, Page 1967, 1970, Fulton 1970, Sawyer 1971, 1975, Menapace et al. 1975, Davis et al. 1978 and Bovee and Sawyer 1979).

Members of the genus *Acanthamoeba* are relatively tolerant of high osmolarities and have often been found in salt water (Page 1981).

Table 1

Seasonal physico-chemical characteristics of sampling belt during the study period (mean of at least three samples)

Seasons	Air Temp. (°C)	Littoral/Interstitial water*				Soil	
		Temp. (°C)	Salinity (°/oo)	Dissolved oxygen (ml/l)	pH	Temp. (°C)	pH
Postmonsoon (1982–1983)	27.23	26.93	20.63	4.18*	8.25	28.00	7.75
Premonsoon (1983)	31.78	30.20	25.13	3.13	8.28	30.80	8.12
Monsoon (1983)	31.10	31.03	18.20	4.65*	8.28*	31.40	7.95
Postmonsoon (1983–1984)	28.10	26.50	20.98	4.22	8.18	27.95	7.92
Premonsoon (1984)	31.94	30.35	26.40	3.34	8.24	31.00	8.04
Monsoon (1984)	31.00	31.02	17.60	4.52	8.22*	31.70	8.00

The present investigation deals with the potency of SDWA media to support the growth of seasonal isolates of *A. rhysodes* from semimarine sandy substrata. These isolates are exposed to the physico-chemical variabilities of the habitat substrata as well as those of neritic water (Table 1).

Materials and Methods

Sampling

Samples (2–6 cm depth) were collected aseptically, transported to the laboratory and processed within 24 h. The exposure period of the sampling belt lasts for 2–3 days per fortnight and the belt remains submerged to depths of about 2–3 m during the rest of the time.

Isolation and Identification of the Strain

Seasonal samples were inoculated with *E. coli* in respective bay water agar (BWA) petridishes and the plates were incubated at 25°C. The amoebae coming from the sample were allowed to grow for a week or more till most of them encysted.

To obtain clonal cultures of *A. rhysodes* from mixed populations of different types of amoebae the following method was used: cysts were put in sterile filtered bay water in cavity slides and diluted to about 40–50 cysts in a slide. A single cyst with typical features of the species in question was picked up by sterile glass micropipette under low power of a microscope and transferred to a bacterial circle in a separate plate for incubation. The amoebae growing from the single cyst were identified as *A. rhysodes* according to Singh and Hanumaiah (1979). Identity of the species was confirmed by reacting the homogenate with anti *A. rhysodes* (AR) serum raised in a rabbit.

For monobacterial culture of *A. rhysodes* isolates, cysts were treated with 2% HCl (W/V) for

2⁴ h and thoroughly washed with sterile filtered bay water under low speed of centrifugation. They were then placed on *E. coli* patch.

Food Source

E. coli was grown on saline adjusted nutrient agar slopes. It was made as follows: To 1 lit of glass distilled water (pH 6.4) 20.0 g Bacto Agar (Difco Laboratories, U.S.A.), 2.60 g Na_2HPO_4, 2.0 g glucose, 20.0 g Bacto Tryptone (Difco Laboratories, U.S.A.) and NaCl as per the salinity of the media were added.

It was then boiled in water bath, dispensed suitably and autoclaved. Routine maintenance of *E. coli* in the laboratory was done with this medium.

Modified Page's Amoeba Saline (PAS) (Page 1966)

To 1 lit of glass distilled water 0.004 g $MgSO_4 \cdot 7H_2O$, 0.004 g $CaCl_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4, 0.136 g KH_2PO_4 were added. NaCl was adjusted as per the salinity of the media. The pH value 8.25, which is the approximate mean value of the seasonal neritic water, was maintained throughout the experiment.

Experimental Methodology

Young cultures of *E. coli* were used as food. Wet weight suspensions of 0.05 g/ml in respective modified PAS were made. Approximately 0.01 g of *E. coli* were added to each of the 5 replicate plates for the individual experimental sets *E* single cysts were inoculated and the plates were incubated at 25°C.

During growth, one can encounter a portion of the organisms without typical trophic or cystic characteristics. These rounded forms without typical locomotory organelle or mature cyst wall are in some way intermediate between the conventional forms. We term them 'Trophocysts'.

To study the growth patterns, the following data were taken into considerations:
(1) Comparison of mean % of trophozoites, trophocysts and cysts of 3, 7, 14, 21, 28, 35 and 42 days of age.
(2) Comparison of trophic and cystic morphology.
(3) Comparison of locomotive rate.
(4) Comparison of 100 days old total population growth expressed in counts/petridish (mean of three replicate plates).

Comparisons of the mean % of trophozoites, trophocysts and cysts were made as follows:
Culture petridishes under microscopic field (100×) reveal that the relative proportion of active (trophic) and inactive (encysted) forms varies consistently between its central zone and periphery during the course of growth.

Initial inoculation was on the centre and excystment followed by rapid multiplication commences here on rapid grazing of the bacteria.

As culture gets old, this zone becomes overcrowded and we call it the 'principal focus' of population growth. Encystment of the organisms here may thus be induced due to depletion of food pH change, waste accumulation etc. Amoebae migrating away from this zone are relatively sparse in their distribution, enjoy available food and maintain their trophic status for comparatively longer period. This constitutes the advancing zones of growth in our study. There are thus two distinct zones of population growth in petridish cultures.

It has often been noted that the migratory amoebae of the most 'advanced region', being sufficiently isolated both spatially as well as from the food zone, do encyst. The data for the two distinct zones of growth have been dealt with separately.

For principal foci areas, media surfaces were aseptically scraped thrice in duplicates at random. On suspending in respective modified PAS about 100–150 organisms per microscopic field (100×)

Table 2

Measurements of strains in different media

Media salinity (°/oo)	Strains	Trophic Morphology								Cystic Morphology			
		Greatest dimension (anterior-posterior) (µm)		Length–width ratio		Nuclear diameter (µm)		Locomotive rate (µm/min)		Diameter (µm)			
										Advancing zone		Principal foci	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
5.0	I	26–30	28	2.20–3.00	2.60	0.70–0.90	0.80	17–32	25	15–30	22	14–24	19
	II	26–30	28	2.15–2.95	2.50	0.70–0.90	0.80	18–31	25	15–30	23	14–24	19
	III	26–31	28	2.24–2.96	2.56	0.70–0.90	0.80	18–32	26	16–30	23	14–24	19
7.5	I	25–31	28	2.20–2.95	2.54	0.70–0.90	0.80	19–32	26	16–30	23	14–23	19
	II	26–30	27	2.15–2.90	2.58	0.70–0.90	0.80	18–32	25	16–29	23	14–24	18
	III	26–31	27	2.15–2.90	2.60	0.70–0.90	0.80	19–30	26	16–30	23	13–24	19
10.0	I	25–30	28	1.94–2.80	2.40	0.70–0.90	0.80	19–33	26	16–28	23	14–24	18
	II	24–30	27	2.00–2.84	2.48	0.70–0.90	0.80	19–32	26	15–30	22	14–23	19
	III	24–31	27	2.10–2.90	2.50	0.70–0.90	0.80	18–32	26	15–29	22	14–23	19
12.5	I	24–30	27	2.10–3.20	2.65	0.65–0.85	0.75	18–30	25	15–30	22	13–24	18
	II	24–30	27	1.98–2.96	2.54	0.65–0.85	0.75	19–31	24	15–28	23	12–23	18
	III	24–31	28	1.94–2.84	2.48	0.65–0.85	0.75	19–30	25	15–28	22	12–23	18

15.0	I	24–30	27	1.80–3.40	2.60	0.65–0.85	0.75	18–32	26	16–30	23	13–23	18
	II	23–30	27	1.92–3.20	2.68	0.65–0.85	0.75	17–30	24	14–28	23	13–23	18
	III	24–30	27	1.88–2.98	2.56	0.65–0.85	0.75	18–31	25	14–29	23	12–24	17
17.5	I	24–30	27	1.84–3.20	2.52	0.65–0.85	0.75	17–30	24	14–27	22	13–21	17
	II	24–30	27	1.86–2.98	2.44	0.65–0.85	0.75	17–29	23	13–27	21	12–22	17
	III	24–31	27	1.90–2.92	2.48	0.65–0.85	0.75	17–29	24	13–29	21	12–23	17
20.0	I	25–30	26	1.88–3.32	2.60	0.65–0.85	0.75	18–30	23	14–29	22	12–23	18
	II	24–30	26	1.74–2.94	2.52	0.65–0.85	0.75	17–28	23	15–30	23	12–24	18
	III	25–30	27	1.88–2.96	2.48	0.65–0.85	0.75	18–28	23	15–29	22	12–23	17
22.5	I	23–28	26	1.82–3.16	2.54	0.65–0.75	0.70	15–28	21	14–26	21	12–20	16
	II	23–30	26	1.84–2.92	2.44	0.65–0.75	0.70	16–28	24	13–26	24	12–22	16
	III	24–28	25	1.92–3.10	2.50	0.65–0.75	0.70	17–29	23	13–27	23	12–22	17
25.0	I	22–29	25	1.94–3.42	2.78	0.65–0.75	0.70	15–29	21	11–27	20	11–22	15
	II	22–28	26	2.00–3.16	2.54	0.65–0.75	0.70	14–27	22	13–25	20	10–22	16
	III	23–28	25	2.20–2.98	2.58	0.65–0.75	0.70	14–28	22	13–25	21	10–21	16
27.5	I	22–29	26	1.98–3.60	2.72	0.65–0.75	0.70	14–28	23	11–25	20	11–20	16
	II	22–28	25	2.12–3.48	2.80	0.65–0.75	0.70	15–28	23	10–26	19	10–21	16
	III	22–28	26	2.24–3.10	2.68	0.65–0.75	0.70	15–27	23	11–24	20	12–20	16
30.0	I	22–27	25	1.90–3.00	2.50	0.65–0.75	0.70	15–29	22	11–23	19	10–20	15
	II	21–28	25	2.10–3.10	2.60	0.65–0.75	0.70	16–28	23	12–26	19	10–21	16
	III	22–28	25	2.00–3.00	2.50	0.65–0.75	0.70	16–29	23	12–24	20	10–21	16

were made and observed immediately. We undertook direct counting approach for the advancing zones. Microscopic fields (100×) containing about 100–150 organisms were selected and a mean of six fields from duplicate plates were considered.

Trophic and Cystic Morphology

Strains harvested from 3-day-old cultures were measured in living condition. Numbers of measurements were: greatest dimension of active amoebae, 100; the other horizontal dimension was measured on 50–100 amoebae to compute length-width ratios; nuclei of amoebae, 25; diameter of cysts, 100. Locomotive forms were observed in hanging drops (Page 1967). Occular micrometer was used for measurements at room temperature (ca. 25°C).

Total Population Growth

After 100 days, plates in triplicates for each set were washed with respective modified PAS, harvested at low speed of centrifugation in 1 ml and counted in Neubaur Haemocytometer. Mean values were expressed in numbers/petridish. It is always possible to lose some of the organisms while harvesting.

The Supporting Media

Saline distilled water agar (SDWA) media. Each of the three sets of experiments require 11 media of different salinity strength ranging from 5–30‰ (W/V). Eleven 1 lit containers were filled with glass distilled water and pH were adjusted to 8.25. To each of the containers NaCl in the quantities 5.0 g, 7.5 g, 10.0 g, 12.5 g, 15.0 g, 17.5 g, 20.0 g, 22.5 g, 25.0 g, 27.5 g and 30.0 g was added separately along with 15.0 g Bacto Agar in each. They were boiled, autoclaved and dispensed in sterile 7.5 cm dia petridishes in five replicates for each medium for three sets of experiments. Media plates were dried for 7 days at room temperature before use.

Bay water agar (BWA) medium. To 1 lit of filtered bay water, 15.0 g Bacto Agar was added. Rest procedures are as above.

Results

Table 2 summarizes the measurements (in μm) of the strains. Mean values of the greatest dimension tend to be smaller in media of increased salinity which may induce effects of hypertonicity resulting in a slight shrinkage in volume (such was the case with nuclear diameters). Such type of relation could not be drawn for the length-width ratios. The locomotive rates of the strains showed slightly decreased mean values as the tonicity increased.

The mean values of the cyst diameter from the two zones of growth tended to be slightly declining as harvests were from media of increased salinity. Comparison reveals that harvests from advancing zones are somewhat greater in diameter with typical morphology, while those from principal foci areas exhibited extensive pleomorphy. The smaller size and morphological deformity probably arose as a result of overcrowding.

Growth curves of all the three strains in different media have been documented. Figure 1 shows the comparison of different media to prove their efficiency to sup-
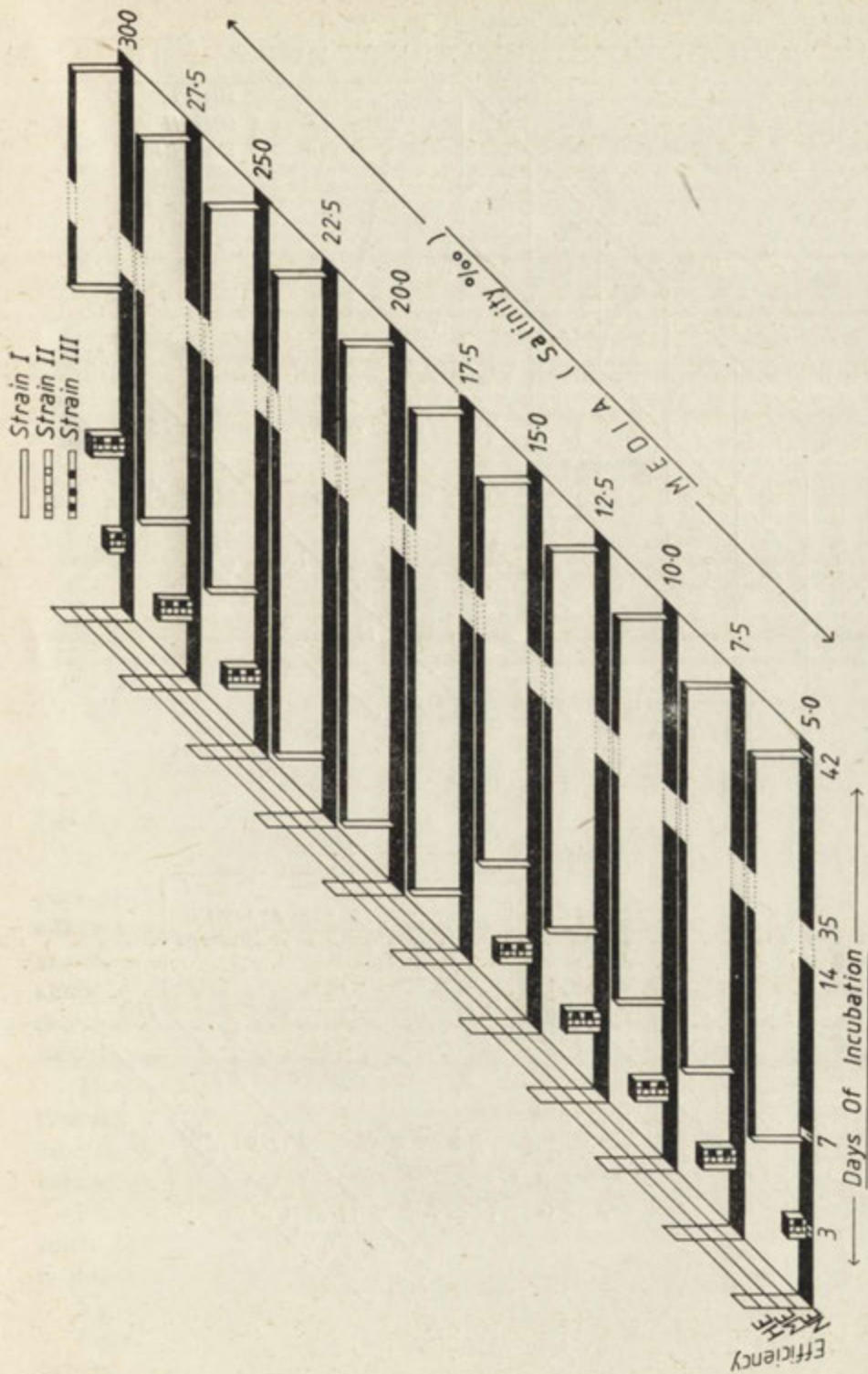
Fig. 1. Efficiency of the media of different salinity gradients to support growth of *A. rhysodes* strains. NE — (Non-efficient media): Average count in six fields (100×) of two replicate plates up to 25 organisms; ME — (Moderately efficient media): This value lies between 25 and 100; HE — (Highly efficient media): More than 100 organisms
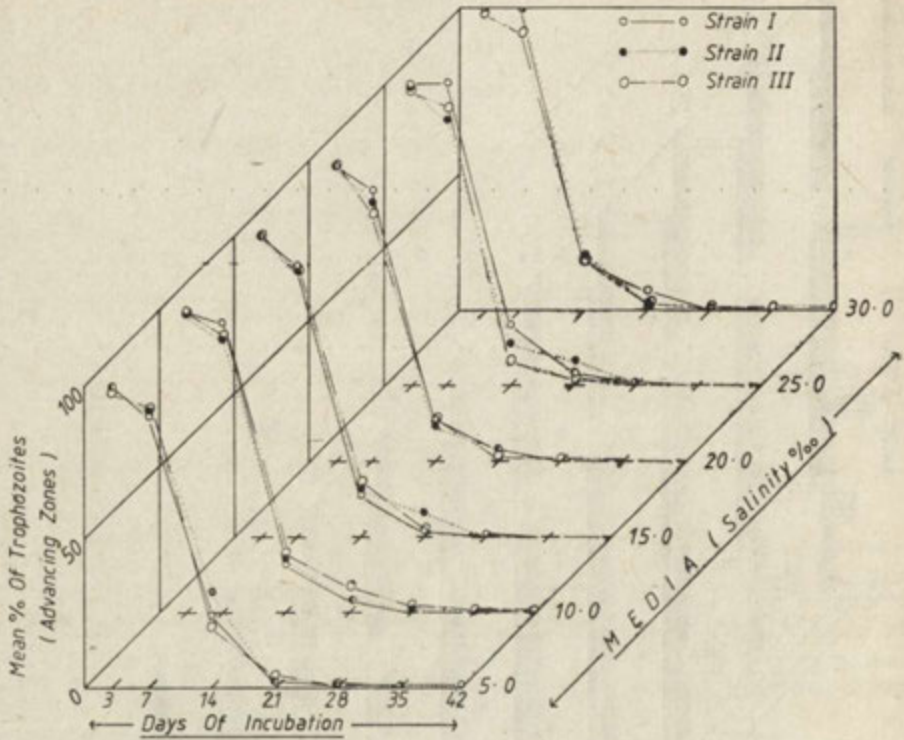
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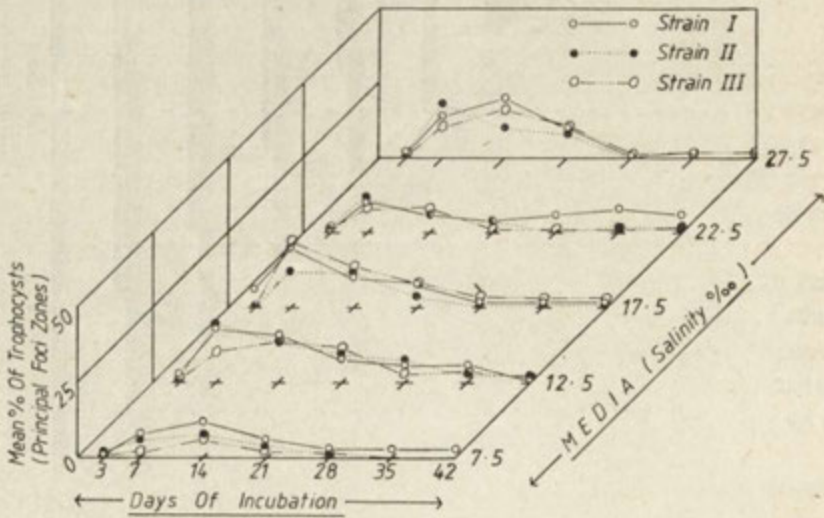
Fig. 3
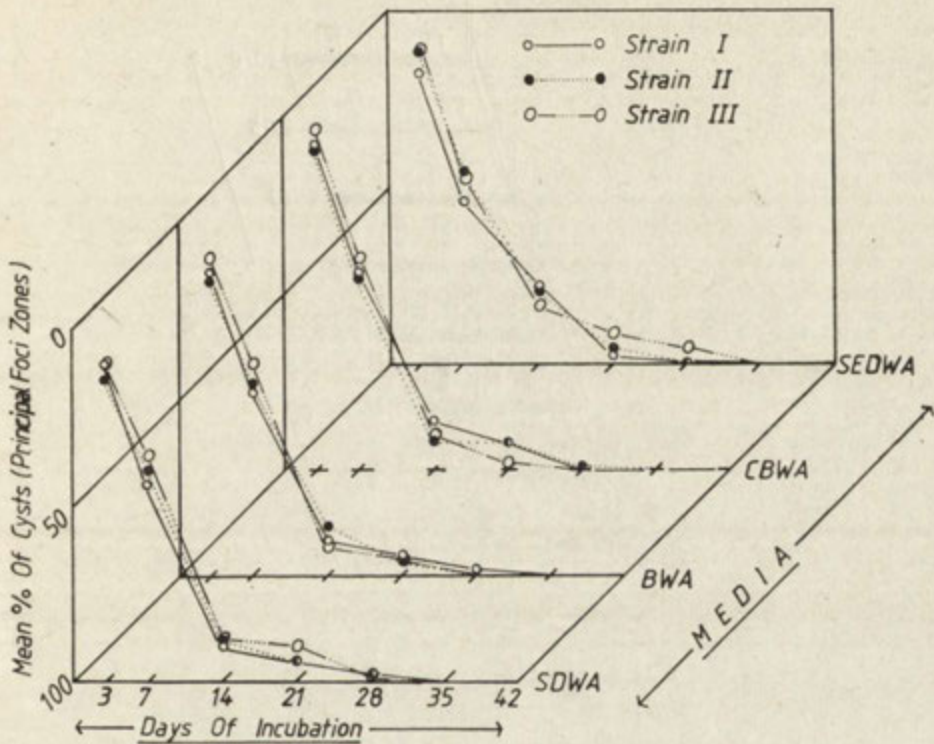
Fig. 4

Figs. 2, 3 and 4. Comparison of the mean % of trophozoites, trophocysts and cysts of the strains of *A. rhysodes* (mean of six fields — 100×, of two replicate plates)

port growth. After 3 days all the strains in this graphic representation exhibit non-efficient to moderately efficient growth in 5–15‰ saline media. Highly efficient growth is shown in 17.5–22.5‰ media and moderate to non-efficient growth again in 25–30‰ media. All media support highly efficient growth on the 7th day and onward, except 30‰ saline medium, which only promote moderately efficient growth till the 7th day.

Thus it becomes apparent that within 7 days the organisms are able to adjust themselves to the osmotic stress, unique for these microbes endowed with such inherent plasticity which probably allows them to react so quickly to sharp environmental changes.

Figure 2 represents the plots for the comparisons of mean percent of trophozoites from advancing zones of growth. Figure 3 presents trophocysts while Fig. 4 is meant for cystic mean percentages.

Figure 3 is for principal foci areas, while Fig. 4 for advancing zones of growth.

Figure 5 represents a bar diagram showing overall growth after 100 days of culture. The average mean values normally range between $1.8–3.4 \times 10^6$ per petri-
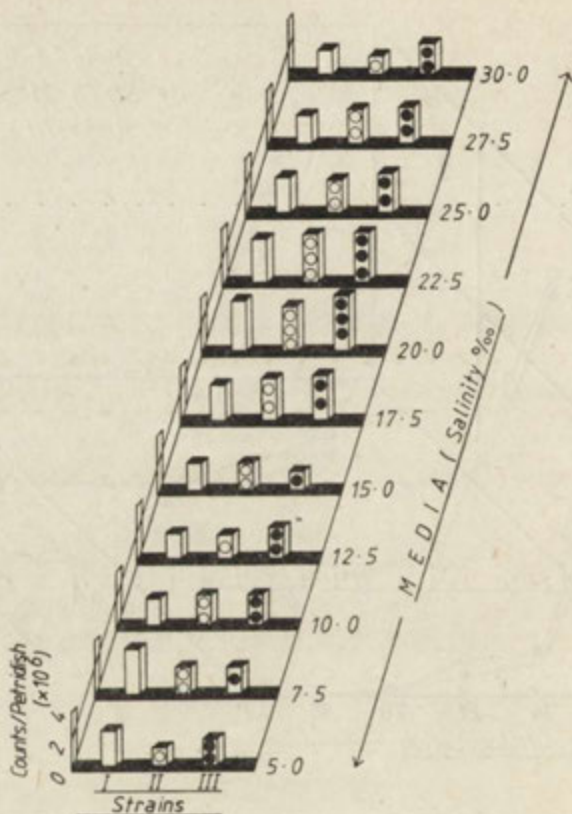
Fig. 5. Overall growth of *A. rhysodes* strains after 100 days of culture in media of different salinity strengths

dish. The greater values are seen to be accumulated in media of intermediate salinity range. Thus media tonicity may not be a very crucial factor controlling the overall growth, once adaptation sets in.

Discussion

The importance of amoebae in the oceanic ecosystems has only recently begun to be assessed. Bamforth (1981) emphasized that being an important epibioict fauna they prey upon phytoplankton and bacteria.

According to Bovee and Sawyer (1979), bacteria eaters and scavengers are important in maintaining the cleanliness of shallow inshore waters and the surface water of open ocean.

Members of the genus *Acanthamoeba* have always been found to be a universal

component of gymnamoebae from the saline environments of lower deltaic Bengal, India, (Bhattacharya et al. 1985, Choudhury and Ghosh 1985, Ghosh and Choudhury 1985 a–c and Ghosh et al. 1985) which is unique in harbouring the luxuriant mangrove ecosystems of the Sundarbans. Preliminary reports on their seasonal abundance in mangrove litter-soils, some aspects of their cultural ecology and their possible role as micropredator components are available in the above references.

It is quite interesting to note that majority of the intertidal benthic fauna acquire a certain degree of euryhalinity as an insurance against fluctuating environmental conditions. Thus a harmonious ecological balance between environmental conditions and the tolerance of an organism to variations in one or more of the said conditions is required for successful development and maintenance of a population.

Here we see that isolates from the same habitats at different seasonal periods with very varied environmental physico-chemical parameters could grow over a full spectrum of salinity in a comparable way.

Comparison of population growth kinetics etc. show that they do not retreat i.e., encyst on challenge with 'salt stress', but adjust and quickly cope up with this new environment. This is in harmony with the idea of Brown and Borowitzka (1979), that a highly permeable membrane, as probably in our cases of naked amoebae, may be of adaptive value to halophilic organisms. A highly saline environment can exert its physiological effect through direct interaction between the salts and cellular constituents; indirectly, through water availability and perhaps through water structure, through osmotically induced pressure and volume changes in a cell (Brown and Borowitzka 1979). These osmophilic organisms residing in the habitats with high osmotic tensions must possess the ability to avoid water loss.

Halophilism or halotolerance can be explained in two aspects of the phenomenon, namely, the ability to survive a transition from one environment to another and the ability to thrive normally in the new environment. According to Brown (1976) the adjustment of a cell to a changed level of salt concentration occurs in two phases followed by a fully adapted steady state situation.

Phase-1

When a cell is transferred to a different level of salinity, there is first a rapid thermodynamic adjustment to the new situation which involves a water flux and a transient osmotic stress.

Phase-2

If the above cell survived, it enters a period of adaptation, the gross physical esult of which will be reversion to a close approximation of its original volume and state of turgor — the physiological readjustment.

Phase-3

It is the fully adapted steady state condition of growth under the new condition.

Thus whether or not an organism should be considered as halophilic or halotolerant, depends on its performance in Phase 3, that is, its ability to grow normally in a highly saline environment.

Further evaluation of the media used in the present investigation, against other selected and widely applicable ones in relation to trophic and cystic morphology and the overall growth has been presented in the accompanying paper II. We would like to propose, finally, that though all the described media possess the ability to support growth in almost unified way, those chosen to be of use should exhibit their efficiency in the shortest time period. It is also hoped to be a good practice to avoid media from either of the extremities.

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Acanthamoeba rhysodes (Singh, 1952) (*Protozoa: Gymnamoebia*) from Gangetic-estuary, India. II. Population Growth Kinetics in Four Selected Media with Reference to the Simplified One

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Synopsis. Acanthamoeba rhysodes (Singh, 1952) strains (accompanying paper I) were grown in four selected media, namely, 20‰ saline distilled water agar (SDWA), bay water agar (BWA), cerophyl bay water agar (CBWA) and soil extract distilled water agar (SEDWA). The growth patterns were compared on the basis of the ability of the media to support highly efficient growth at an early period, morphometry of trophozoites and cysts from different media and the overall growth after 100 days of culture. All the media were equally competent in inducing highly efficient growth and there was uniformity in measurements of strains from different media. Maximum growth, however, was encountered in BWA medium, followed by SDWA, SEDWA and CBWA media in sequence.

The population growth kinetics of the premonsoon, monsoon and postmonsoonal isolates of *A. rhysodes* (referred to as strains I, II and III in sequence as in accompanying paper I) in a wide spectra of SDWA media have already been presented (accompanying paper I). Though not a single medium can be considered to be natural, using one or more of the ingredients available in the habitat of the isolated organisms in the culture medium would certainly make it nearer to a natural situation. In SDWA medium we have no such component other than NaCl, a universal constituent of saline environments, and thus one might question about the probable pleomorphy of cultured organisms that resulted due to the media themselves. It is with this idea we have planned to study the population growth kinetics in comparable ways, using media of different types having at least one of the ingredients from the habitat itself, namely, BWA, CBWA and SEDWA media and the devised SDWA media (accompanying paper I).

Materials and Methods

Amoeba Strain

The strains of *A. rhysodes* are those isolated during the premonsoon, monsoon and postmonsoonal periods. Isolation and establishment of clones are the same as in accompanying paper I.

Food Source

Laboratory maintained *Escherichia coli* has been used as food.

Details of maintenance are given in the accompanying paper I.

Modified Page's amoeba saline (PAS) (Page 1966). For studies with SDWA medium a modified PAS was preperad as follows: To 1 lit of glass distilled water the following ingredients were added: 20.0 g NaCl, 0.004 g $MgSO_4 \cdot 7H_2O$, 0.004 g $CaCl_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4, 0.136 g KH_2PO_4.

For other media, all the chemicals others than NaCl were added to respective fluid bases.

The Supporting Media

The simplified one: Saline distilled water agar (SD WA) medium. To 1 lit of glass distilled water 20.0 g of NaCl, 15.0 g of Bacto Agar (Difco Laboratories, U.S.A.) were added, boiled in water bath, autoclaved, and the molten medium was dispensed in 7.5 em dia petridishes in replicates for 3 sets. pH was adjusted as before (accompanying paper I).

Bay Water Agar (BWA) Medium

To 1 lit of filtered, habitat associated seasonal water samples from the littoral zone 15.0 g of agar was added, boiled and autoclaved. Table 1 (accompanying paper I) shows their salinity and pH values. pH of the medium was adjusted as before (accompanying paper I).

Cerophyl Bay Water Agar (CBWA) Medium

Prepared after Page (1970) with minor modifications. One g of cerophyl (Cerophyl Lab. Inc., U.S.A.) was boiled in 1 lit of filtered water as BWA medium, filtered thrice through glass wool. Fifteen g of agar was then added, boiled and autoclaved. pH was adjusted as before (accompanying paper I).

Soil Extract Distilled Water Agar (SEDWA) Medium

Prepared after Singh (1975) with slight modification. 400.0 g of seasonal substrate samples were boiled in 1 lit of glass distilled water for 30 min, extracts were decanted, filtered and finally made to 1 lit. 15.0 g of agar was added to each medium, boiled and autoclaved. pH was adjusted as before (accompanying paper I).

Experimental Methodology

Final wet weight suspensions of 0.05 g/ml of *E. coli* were made in respective modified PAS. To five replicate plates for each medium for all the sets of strains, suspensions were added, so that each gets about 0.01 g wet weight of *E. coli*. Inoculation of cyst, and data collections were as before (accompanying paper I).

Results

All the media exhibit highly efficient growth within 3 days of culture.

Individual measurements from different media are summarized in Table 1. As in the accompanying paper I, cyst diameters tend to be smaller from principal

Table 1

Measurements of the strains in different media

Media	Strains	Trophic morphology								Cystic morphology			
		Greatest dimension (anterior-posterior) (μm)		Length-width ratio		Nuclear diameter (μm)		Locomotive rate (μm/min)		Diameter (μm)			
										Advancing zone		Principal foci	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
SDWA	I	22–31	28	1.80–3.40	2.50	0.65–0.85	0.75	18–30	24	14–27	22	13–23	20
	II	23–30	27	1.80–3.35	2.48	0.65–0.85	0.75	18–30	23	15–29	21	12–25	20
	III	22–30	27	1.75–3.00	2.25	0.65–0.85	0.75	17–31	24	15–29	22	13–25	19
BWA	I	23–31	27	1.74–3.20	2.35	0.65–0.85	0.75	17–29	23	15–29	22	12–23	19
	II	24–30	28	1.84–3.36	2.52	0.65–0.85	0.75	18–32	24	16–29	23	12–23	18
	III	24–30	27	1.80–3.40	2.50	0.65–0.85	0.75	18–30	25	16–30	23	13–24	19
CBWA	I	23–30	27	1.88–3.44	2.46	0.65–0.85	0.75	17–29	24	16–30	21	13–26	19
	II	23–29	26	1.90–3.32	2.52	0.65–0.85	0.75	17–31	24	15–30	22	14–24	18
	III	24–31	27	1.86–3.40	2.36	0.65–0.85	0.75	17–30	26	15–29	22	12–22	20
SEDWA	I	24–31	27	1.90–3.20	2.54	0.65–0.85	0.75	18–28	24	15–30	22	13–25	18
	II	23–29	27	1.88–3.32	2.48	0.65–0.85	0.75	18–31	25	16–28	22	13–23	18
	III	24–30	28	1.82–3.36	2.28	0.65–0.85	0.75	17–30	23	16–28	22	13–23	17
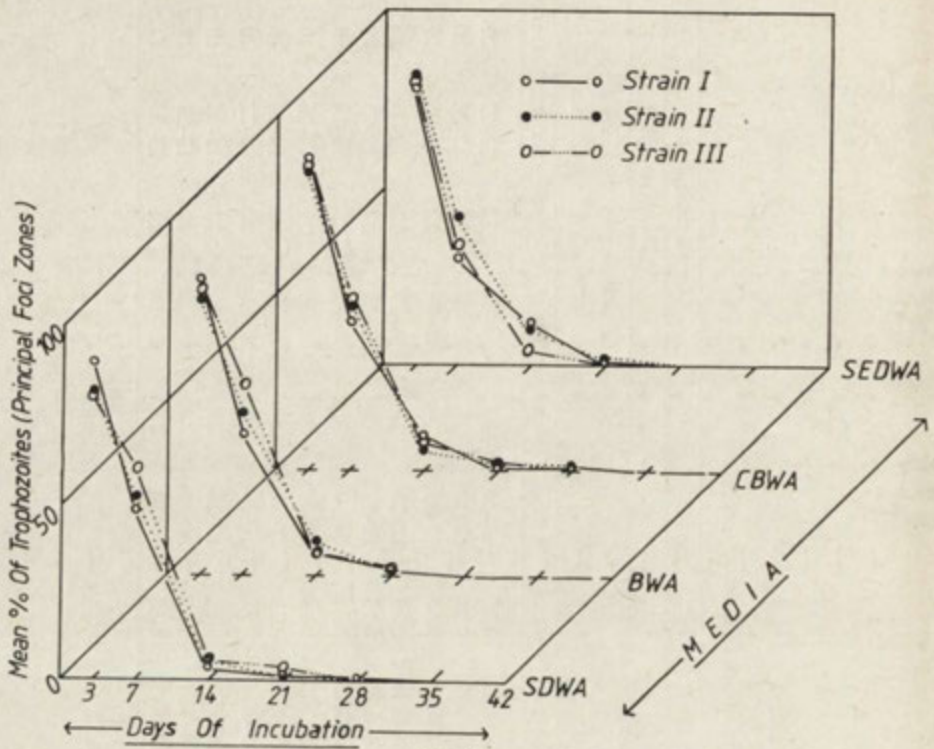
Fig. 1
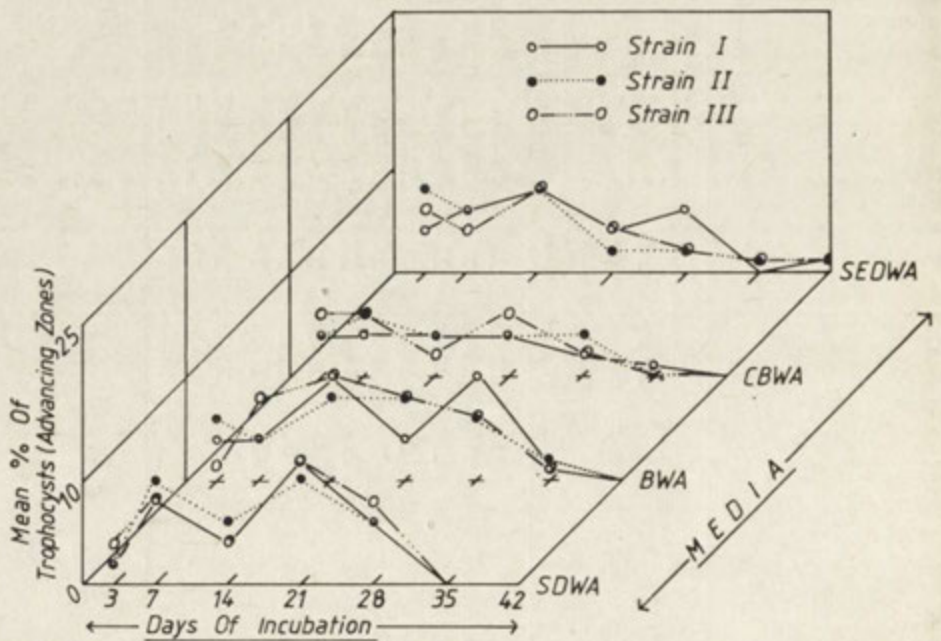
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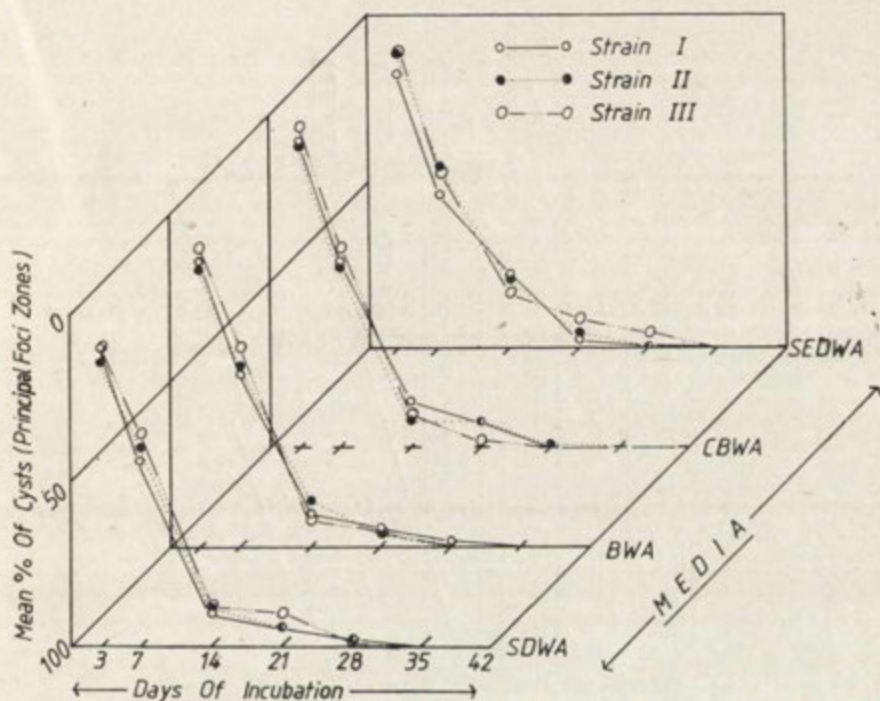
Fig. 3

Figs. 1, 2 and 3. Comparison of the mean % of trophozoites, trophocysts and cysts of *A. rhysodes* strains (Mean of six fields — 100×, of two replicate plates) in four selected media

foci of growth than those from the advancing zones. Figure 1 represents the mean % of trophozoites from the principal foci areas. Figure 2 depicts trophocysts, while Fig. 3 is meant for cystic mean percentages. Figure 2 is for advancing zones, while Fig. 3 for principal foci of growth. Overall quantitative growth, as bar diagram, is presented in Fig. 4. The highest growth was in BWA medium followed by SDWA, SEDWA and CBWA media. The growth is maximum for strains II and III in BWA medium and minimum for the same strains in CBWA medium. No sharp discrimination has been observed as of the growth patterns of strains under investigation, preferring one medium or the other.

Discussion

Population growth kinetics of the seasonal isolates of *A. rhysodes* clearly demonstrate that aerobic naked amoebae, isolated from diversified saline environments can very well be cultured in SDWA media of wide range, from as low as $5^0/_{00}$ to as high as $30^0/_{00}$. Thus to initiate cultures from samples lacking adequate chemical parameters, the most important of which is salinity, media of wide spectral salinity values need to be tried. It is possible to predict the moderate tolerable li-
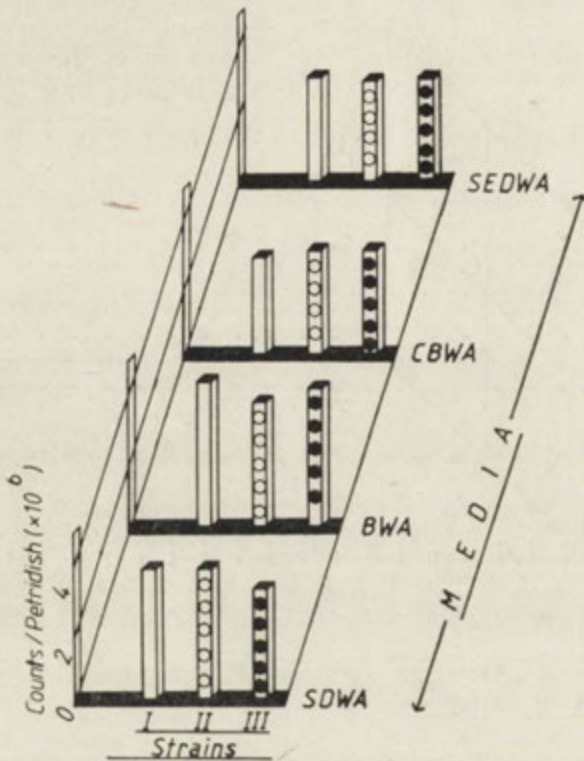
Fig. 4. Overall growth of the strains under investigation after 100 days of culture in the selected media

mits within two weeks of culture. The main objective of establishing and evaluating such a medium of minimal necessity is to develop a method to culture this important component of soil microbiota from saline environments in the simplest way. Studies on their growth patterns in relation to other environmental parameters, both abiotic and biotic are under way.

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A New Myxosporidian, *Parazschokkella melanosticti* gen. n., sp. n. Parasitic in the Gall Bladder of the Toad, *Bufo melanostictus*

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Synopsis. A new myxosporidian, *Parazschokkella melanosticti* gen. n., sp. n. is described from the gall bladder of the toad, *Bufo melanostictus* and there are no external indications of infection. Fresh spores were brownish in colour and semi circular in front view. In a side view they had a characteristic fusiform shape, both ends being bluntly rounded with lid-like structures. They are broader than long and measured $6.5 \times 9.8 \times 6.2$ μm in the fresh condition. The two spore valves were fused along a thick raised longitudinal sutural ridge which was slightly arched. Each spore valve showed 8–12 deep transverse striations. The polar capsules were widely separated and were placed at opposite poles. Polar capsules were oval in shape measuring 2.2×1.8 μm. The polar filament showed 2–3 coils inside the polar capsule and when fully extruded measured 26–30 μm in length and showed a deeply basophilic cushion-like structure at the base. The filament was ribbon-shaped and was club-shaped distally. An iodinophilous vacuole was not observed.

Myxosporidians are generally parasites of fishes invading the scales, skin, gills and various internal organs. They are rarely found as parasites of amphibians and reptiles. While examining the toad, *Bufo melanostictus* for myxosporidian parasites, we came across a new myxosporidian infecting the gall bladder of the toad which is described in the present communication.

Material and Methods

Toads were collected from the fields at night with the help of a small net after making them blinded by focussing a torch light on their eyes. A total number of 394 toads were collected from the fields from 3 different towns in Andhra Pradesh, India (Visakhapatnam, Vizianagaram and Machilipatnam). Soon after they were brought to the laboratory they were anaesthetized, dissected and various internal organs examined for myxosporidian parasites. Two of the 58 *Bufo melanostictus* collected from Machilipatnam showed infection in the gall bladder. When infected the gall bladder could be identified easily because of the colour and consistency. Conventional methods of fixing and staining were used to study the parasites. All the drawings were made with the aid of a camera lucida and the measurements of the spores given in microns (μm) were those suspended in normal saline.

Observations

Parazshokkella melanosticti gen. n., sp. n.

Host: *Bufo melanostictus*
Site of infection: Gall bladder
Locality: Machilipatnam, Andhra Pradesh, India
Type slides: Authors' Collections and Department of Zoology, Andhra University, Waltair, India

Diagnosis of species

Trophozoites coelozoic floating in the bile. Multinucleate plasmodia with 6–12 nuclei measured $5.0–9.5 \times 3.8–7.5$ μm. Pansporoblasts free in the lumen, disporic and measured $20–25 \times 10–15$ μm. Spore semicircular in front view with broad flat anterior and rounded posterior ends, fusiform with bluntly rounded ends in lateral view, broader than long and measured $6.0–7.0$ $(6.5) \times 9.6–10.5$ $(9.8) \times 6.0–6.4$ (6.2) μm in the fresh condition. Small lid-like structures were seen at the rounded corners which may be thickenings of the spore valves. Spore valves fused along a thick raised arched sutural ridge. Spore valves showed 8–12 deep transverse striations. Polar capsules at opposite poles, oval in shape and measured $2.0–2.5$ $(2.2) \times 1.6–2.0$ (1.8). Polar filament showed 2–3 coils in the capsule and when fully extruded measured 26–30 μm in length. Filaments ribbon-shaped and were club-shaped distally. A strongly basophilic cushion-like structure was seen at the base of the filaments. Parasites of toad — *Bufo melanostictus*.

Life-cycle

The trophozoites were coelozoic and were freely floating in the bile. The earliest stage of the parasite observed was binucleate measuring 3.0–3.2 μm (Fig. 1 *1*). The cytoplasm was alveolar and the nuclei were homogenously deeply stained. Multinucleate plasmodia with 6–12 nuclei measured $5 0–9.5 \times 3.8–7.5$ μm (Fig. 1 *2*). The nuclear structure was similar to those of the previous stages. The nuclear material in the trophozoites showed condensation of the chromatin and differentiation of two types of nuclei in the later stages of development. Some of the nuclei were small in size with a single irregular chromatin mass and an incipient nuclear membrane. The second type of the nuclei were those with a well defined nuclear membrane and having one or two chromatin granules adherent to the nuclear membrane (Fig. 1 *3* and *4*). Pansporoblasts measuring $20.0–25.0 \times 10.0–15.0$ μm were found in the bile and they were disporic (Fig. 1 *5* and *6*). The developing sporoblasts showed 2 sporoplasmic, 2 capsulogenous and 2 valvogenous nuclei (Fig. 1 *7*). Spores were semicircular in front view and had a broad flat anterior and rounded posterior end (Fig. 1 *9* and *10*). They were broader than long and measured 6.0–7.0
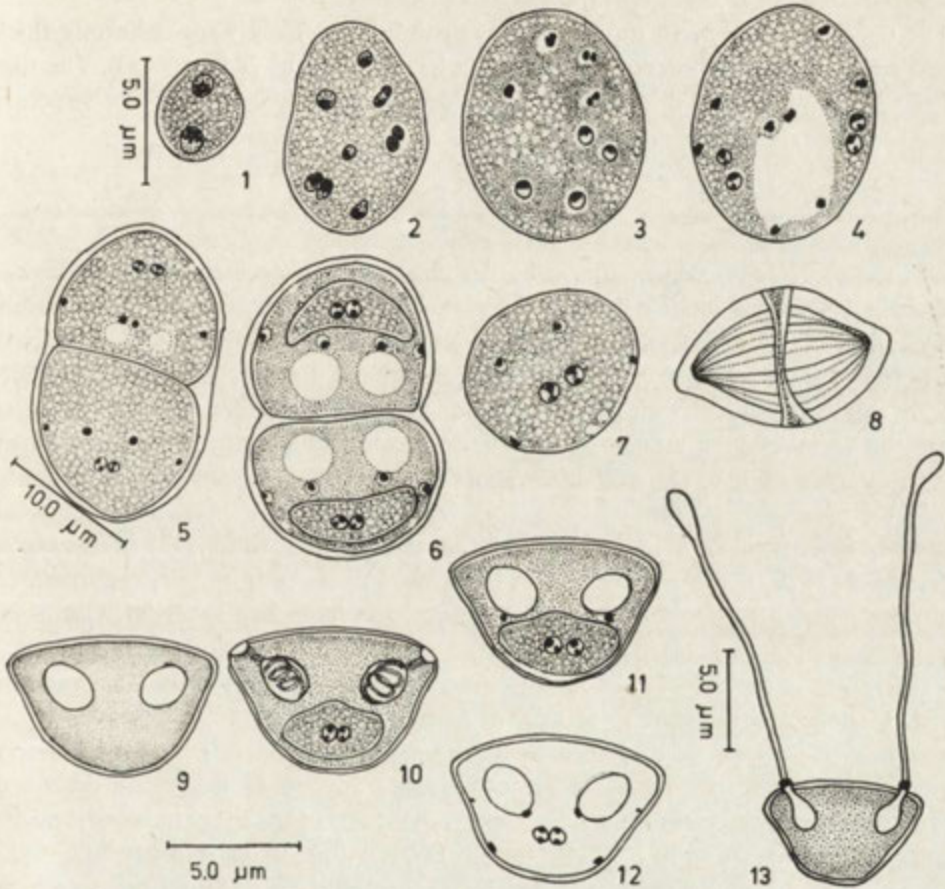
Fig. 1. *1* and *2* — Trophozoites, *3* and *4* — Sporogonial plasmodia showing generative and somatic nuclei, *5* and *6* — Disporic pansporoblasts, *7* — Developing sporoblast, *8* — A fresh spore-lateral view, *9* — A fresh spore, *10* — Spore stained with Giemsa, *11* — Spore stained with iron haematoxylin, *12* — Spore stained according to Feulgen's technique, *13* — Spore with extruded polar filaments

(average 6.5, n = 50)×9.6–10.5 (average 9.8, n = 50)×6.0–6.4 (average 6.2, n = 50) in the fresh condition. Small lid-like structures were seen at the rounded corners. The spore valves were fused along a thick, raised longitudinal sutural ridge which was slightly arched. Each spore valve showed 8–12 deep striations extending transversly (Fig. 1 *8*). The polar capsules were widely separated and were placed at opposite poles. They were oval in shape and measured 2.0–2.5 (average 2.2, n = 50)× ×1.6–2.0 (average 1.8, n = 50). A fine duct extended from the polar capsule towards the lid-like structure. The polar filament showed 2–3 coils inside the capsule (Fig. 1 *10*). The sporoplasm was amoeboid and binucleate. The nuclei had 1–2 deeply stained granules adherent to the nuclear membrane (Fig. 1 *11*). The polar filaments were extruded when treated with saturated aqueous urea or 2.5% KOH.

The polar filaments were extruded through the ducts. They were uniformly thick and ribbon-like and measured 26–30 μm in length (average 28.0, n = 50). The filament was club-shaped distally and had a deeply basophilic cushion-like structure at the base.

Taxonomic Position

Tripathi (1948) created the suborder *Bipolarina* to accommodate the myxosporidian parasites having "two polar capsules at opposite ends or with widely divergent polar capsules located in the sutural plane or sutural zone" (Quoted from Levine et al. 1980). This suborder at present includes 4 genera (1) *Myxidium* Büetschlii: spores fusiform with pointed or rounded ends, polar capsules one at either end, no iodinophilous vacuole, sutural line typically straight and coincides with or is at an acute angle to the axis of the spore, polar filament filamentous not ribbon-like. (2) *Spheromyxa* Thelohan. Spore fusiform with truncate ends. Polar filament ribbon-like. Trophozoites discoidal coelozoic in marine fishes. (3) *Zschokkella* Auerbach, 1910. Spore semicircular in front view, fusiform in profile, ends pointed obliquely, sutural ridge "S" shaped. Polar capsules large and spherical. Coelozoic in fish or amphibians. (4) *Sinuolinea* Davis, 1917. Spore spherical or subspherical with a sinuous sutural line. Polar capsules some distance away from the anterior end. Coelozoic in the urinary bladder of marine fishes.

The present form is a typical bipolarine myxosporidian in having two widely seperated polar capsules, but it does not resemble anyone of the genera described in all its features. It resembles *Zschokkella* in the shape of the spore in being semicircular in front view and fusiform in lateral view, but differs from it in having a slightly arched and raised sutural ridge instead of the "S" shaped sutural ridge seen in *Zschokkella*. The present form shows some resemblances to the genus *Sphaeromyxa* particularly in having a short ribbon-like polar filaments. The shape of the spore, however, is considerably different, being fusiform with truncate ends. The present form also has ribbon-like filaments but with club-shaped distal ends and strongly basophilic cushion-like structures at the base. It does not show any resemblance to either *Myxidium* or *Sinuolinea*.

Chakravarty (1940) reported in the spores of *Zschokkella lissemysi* from the gall bladder of *Lissemys punctatus* a lid-like structure in the inter capsular region into which the polar capsules opened through a fine duct running at an acute angle and converging in the middle of the inter capsular region. He believed that the sporoplasm flowed out from the polar capsule, through the duct to the outside through the opening when the lid-like structure opened. The lid-like structures in the present form may be homologous to those described by Chakravarty (1940) with the difference that in the present form the polar capsules were divergent and hence open independently at the poles. Chakravarty (1940) had not described a sutural ridge, much less an "S" shaped one which is characteristic of *Zschokkella*.

Z. auerbachi (Weill) is the only foim reported from amphibian hosts, *Bufo melanostictus, Rana tigtina* and *R. limnocharis.* The present form is observed only in toad *Bufo melanostictus.* Frogs *Rana tigrina, R. limnocharis, R. breviceps* and *R. cyanophlyctis* collected from the same and nearby areas did not show the infection. Although the spores in the present form and *Z. auerbachi* resemble each other in size, they differ considerably in other features. The sutural ridge in *Z. auerbachi* is oblique and it is arched in the present form. The trophozoites are polysporous in *Z. auerbachi,* while they are disporous in the present form. The present form is unique in having semicircular spores with a lid-like structure each at either pole, the corresponding polar capsules opening near the lids through a fine duct. The polar filaments are flat and ribbon-like with club-shaped distal ends and having deeply stained cushion-like structures at the base. In view of what is stated, it seems necessary to create a new genus to accommodate the present parasite and the name *Parazschokkella melanosticti* gen. n., sp. n. is proposed for the same. It is further proposed to transfer the two species, *Zschokkella auerbachi* (Weill) from *Bufo melanostictus, Rana tigrina* and *R. limnocharis* and *Z. lissemysi* Chakravarty from *Lissemys punctatus* to the new genus *Parazschokkella,* which will then accommodate the parasites of amphibians and reptiles, while the genus *Zschokkella* accommodates the species parasitic in fishes.

Diagnosis of genus *Parazschokkella* gen. n.

Bipolarine myxosporidian. Spore semicircular in front view and fusiform in lateral view. Sutural ridge slightly arched with raised edges. Spore valves striated with a lid-like structure at the opening of the polar capsules. Polar capsules oval or spherical either divergant or convergent. Polar filament thick and ribbon-like. Parasites of amphibians and reptiles,

Type species: *Parazschokkella melanosticti* sp. n.

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