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ACTA PROTOZOOLOGICA

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Numerical Taxonomy of *Blepharisma* Based on the Effects of Selected Antibiotics

Synopsis. Ten stocks of *Blepharisma* were cultured in separate solutions of streptomycin, penicillin and tetracycline for 96 h. Survival and growth rates for each stock in each antibiotic were determined. After four days survivors were fixed, stained and measured for morphological characteristics. Survival, growth rates and morphological characteristics were coded for the methods of numerical taxonomy. Computer analysis was performed to determine overall similarities among the stocks. The stocks clustered in similar groups when physiological and morphological characteristics were analyzed. The physiological data provided additional information useful in *Blepharisma* taxonomy and in classifying controversial species named in the literature.

Blepharisma is a red pigmented, heterotrichous ciliate found in fresh and marine environments. The genus *Blepharisma* was created in 1849 by Perty. Since then the genus has been described by numerous investigators (Calkins 1912, Woodruff and Spencer 1922, Moore 1924, Stolte 1924, Woodruff 1935, Giese 1938, Weisz 1949, 1950a, b, 1954, Suzuki 1951, 1954, 1957, Hirshfield and Pecora 1956, McLoughlin 1957, Helson et al. 1959, Christie 1965, Kennedy 1965, Dembitzer and Hirshfield 1966).

"Morphological considerations continue to form the basis of systematics in protozoology... Physiological, or biochemical, genetic, ecological and morphogenetic factors are becoming of increasing importance as our knowledge grows in these principally experimental areas of research" (Corliss 1959). With more extensive use of the electron and scanning microscopes and with more sophisticated biochemical and genetical studies, taxonomy is becoming increasingly more precise.

The genus *Blepharisma* is fairly well defined, but classification of many of the named species within the genus is still controversial. Previously, speciation was based primarily on the size of the vegetative animal, the form and shape of the macronucleus and its behavior during bi-

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nary fission and conjugation (Calkins 1912, Woodruff 1935, Giese 1938, Weisz 1950a, b, Suzuki 1957, Bhandary 1960, 1962, Seshachar and Bhandary 1962, Jenkins 1967), although other characteristics have also been considered (Stolte 1924, Dawson 1929, Giese 1938, Young 1939, Hirshfield and Giese 1953, Suzuki 1954, McLoughlin 1957, Bhandary 1959, Helson et al. 1959, Seshachar et. al. 1959, Hirshfield et al. 1962, Seshachar and Bhandary 1962, Bhandary and Hirshfield 1964, Chunosoff et al. 1965, Christie and Hirshfield 1967, Repak 1968). Only a few physiological studies of *Blepharisma* have been reported (Stolte 1924, Helson et al. 1959, Giese and McCaw 1963, Christie 1968, Giese 1970), and, of these, none has examined the usefulness of physiological characteristics as a taxonomic tool.

A numerical taxonomic system can help resolve some of the confusion pertaining to the classification of the species and sub-species of *Blepharisma* by the use of "arithmetical methods to classify organisms on the basis of their overall similarity to one another" (Liston 1970). This paper reports the application of numerical taxonomy to ten stocks of *Blepharisma* based on the effects of streptomycin, penicillin and tetracycline on the survival and growth of the organisms and on the stability of the morphological characteristics.

The use of physiological characteristics in the system of computerized numerical taxonomy introduces a new approach to speciation, and a new dimension to the classification of *Blepharisma*.

Materials and Methods

The *Blepharisma* used in this study were cloned from New York University laboratory stocks. Weekly subcultures were maintained on 0.3% Cerophyl and a *Pseudomonas ovalis* inoculum, and were dark grown at 22°C.

The *Blepharisma* [1] selected were:

Binodal Macronucleus

B. tropicum Bhandary; New Delhi, India Strain

Multinodal Macronucleus

B. americanum Suzuki; Monterey, California Strain

B. americanum Suzuki; Stanford, California Strain

B. musculus v. *seshachari* Bhandary; Lake Stella, Washington Strain

B. musculus v. sinuosum Sawaya, Bangalore, India B Strain

Filiform Macronucleus

B. japonicum Suzuki; Cameroun, Africa Strain

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[1] As classified by Hirshfield H. I., Isquith I. R. and DiLorenzo A. M. Classification, Distribution and Evolution. In *Blepharisma* (ed. A. C. Giese). pp. 304–332, Stanford Univ. Press, Stanford, California (1973).

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B. japonicum Suzuki; Yamagata, Japan Strain, Suzuki 5 Stock
B. japonicum v. *intermedium* Bhandary; Bangalore, India A Strain, red Stock
B. japonicum v. *intermedium* Bhandary; Bangalore, India A Strain, albino Stock
B. stoltei Isquith; Lake Federsee, Germany Strain, albino Stock.

Three day old stock cultures in log phase were used for all experiments. *Blepharisma* were washed three times in sterile 0.1% Cerophyl. Ten organisms were placed into each well of a 9-hole, deep well plate containing 1 ml of a 10^{-5} Molar solution of Streptomycin Sulfate, Penicillin G Potassium or Tetracycline Hydrochloride (Cal Biochem, Los Angeles, California). The 10^{-5} Molar antibiotic cencentration retarded the division of the *Blepharisma* without producing lethai effects. Antibiotics were dissolved in sterile 0.1% Cerophyl and filtered through a 0.45 millipore plate. A 0.1% Cerophyl solution was used as the control.

Each plate was put into a moisture chamber, sealed and placed in the dark at 22°. Daily counts of the surviving number of organisms in each well were made. Experiments for each drug were repeated a minimum of four times (36 trials). Each trial lasted four days and a mean experimental/control survival and growth ratio was determined for each stock in each of the three antibiotics.

After 96 h the organisms were fixed in Carnoy's fixative and stained by the Feulgen-light green reaction or with Methyl green-pyronin Y (Gurr 1966). The morphological characteristics observed and measured for each of the ten stocks in the three antibiotics and the control included body length, body width, shape of anterior end, macronuclear shape, presence or absence of macronuclear nodes, size of largest macronuclear node, presence of equal node size, filiform knobs on macronucleus, length of macronucleus, width of macronucleus, degree of macronuclear bending, presence of macronuclear connecting strands, degree of pigmentation, cavities in macronucleus, and buccal length.

The data were converted into a series of positive and negative answers using Methods I and V according to Lockhart (1970), and were key-punched onto standard IBM cards. The program in Fortran IV was designed and written by L. Neil Bell (Berkeley, California) and utilized a CDC 6600 computer (New York University's Courant Institute of Mathematical Sciences). The data were analyzed by simple matching which considers similarity between two positive characters as well as similarity between two negative characters. The cluster analysis was performed by means of the single-link method (Sokal and Sneath 1963). As designed, the program can evaluate and determine certain coefficients of resemblance and also perform cluster analysis of these coefficients. When all the clusters were completed, dendrograms were constructed showing the level of similarity among the ten stocks.

Results

Bacterial Studies

In nature, *Blepharisma* are bacterial feeders (Stolte 1926, Sandon 1932, Dawson 1928, 1953). Only a few strains of *Blepharisma* have been successfully grown axenically on undefined medium (Giese 1973). In general, growth and division rates for axenic *Blepharisma* are usually

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lower than those for the bacteria-fed controls and growth under these conditions can only be sustained for a limited period. For this reason, *Pseudomonas ovalis* bacteria were used as a food supplement in these studies.

Since certain antibiotics interfere with the life processes of some bacteria, it was important to determine how much of an effect the selected antibiotics have on the bacteria and to what extent this influences the survival of *Blepharisma.*

Pseudomonas bacteria were grown in test tubes containing 10^{-4} M, 10^{-5} M and 10^{-6} M concentrations of each of the three antibiotics and in a control test tube containing 0.1% Cerophyl. Serial dilutions ranging from 1/10 to $1/10^6$ were made of each concentration for each of the drugs and the control for four consecutive days. The dilutions were pipetted into test tubes containing nutrient agar and poured into sterile petri dishes. The number of bacterial colonies that grew on the agar plates after 24 h was counted. Only colony numbers between 30 and 100 per plate were considered significant. Bacterial growth was also tested with antibiotic sensitivity disks (Difco Laboratories, Detroit, Michigan).

The results of these dose response studies indicated that the *Pseudomonas* are only slightly sensitive to the drugs at the 10^{-5} M concentrations used in this research. At this concentration, the bacteria are able to survive and grow, and therefore the effect on the survival of the *Blepharisma* is not a result of cessation of growth of the food organism.

Additional tests, in which a 0.1 ml drop of *Pseudomonas* culture was added daily to the experimentals and controls, showed that neither the drug-treated *Blepharisma* nor the control organisms survive any better with a supplement of fresh bacteria.

Physiological Results

Table 1 summarizes the results for each stock in the antibiotics. The "Experimental" number is the mean number of surviving experimental organisms per 96 h experiment, and was determined by dividing the total number of survivors by the number of experiments. The "Control" number was similarly obtained from cultures lacking antibiotics.

The Experimental /Control ratio (%E/C) indicates the percent survival and growth for each stock, and was obtained by dividing the mean number of surviving experimental organisms by the mean number of surviving control organisms (Table 1). The %E/C ratio evaluates the individual division rate differences inherent within the stocks. These division rates are represented on Table 2.

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Table 1
Survival and Growth After 96 h in Antibiotics

Stock	Experimental[2]	Control[2]	%E/C[2]
Streptomycin			
Bangalore A red	536	778	68.9
Lake Federsee albino	143	309	46.2
Cameroun	150	507	29.7
Lake Stella	185	669	27.7
Yamagata	199	816	24.4
Bangalore A albino	132	553	23.9
Bangalore B	212	901	23.5
Monterey	193	1036	18.6
Stanford	176	1111	15.8
New Delhi	168	1371	12.3
Penicillin			
Bangalore A red	912	711	128.3
Bangalore A albino	401	450	89.3
Monterey	916	1105	82.9
Cameroun	474	586	80.9
Lake Federsee albino	208	262	79.5
Bangalore B	456	725	62.9
Yamagata	386	629	61.4
New Delhi	374	1010	37.0
Lake Stella	203	560	36.3
Stanford	210	706	29.8
Tetracycline			
Bangalore A red	254	602	42.1
Lake Federsee albino	130	346	37.6
Bangalore B	174	582	29.9
Cameroun	151	531	28.4
Bangalore A albino	131	524	24.9
Lake Stella	138	602	22.9
Yamagata	113	661	17.1
Stanford	182	1215	15.0
Monterey	116	1199	9.7
New Delhi	56	1051	5.3

The physiological data can be summarized as follows:

(1) Variation is found in the survival and growth rates among the ten stocks of *Blepharisma* in each of the three antibiotics tested.

[2] See Physiological Results, p. 88, for explanation of the headings.

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Table 2

Control Division Rate/24 h

Stock	Mean division rate[3]
New Delhi	3.2
Monterey	3.1
Stanford	2.8
Bangalore B	2.0
Yamagata	2.0
Bangalore A red	1.9
Lake Stella	1.7
Cameroun	1.5
Bangalore A albino	1.4
Lake Federsee albino	0.9

(2) Of these, penicillin seems to have the least effect. Although exposure to the antibiotics for four days inhibits the growth of the *Blepharisma*, the organisms fully recover from the effects of the drugs when removed and placed into 0.1% Cerophyl. These survivors were not found to be more resistant to the antibiotics when reinoculated into the drugs a second or third time, suggesting that no selection for drug resistance occurred.

Computer Results

Two major groups of *Blepharisma* are delineated when simple matching similarity coefficients of the ten stocks are clustered. Group 1 is composed of the Cameroun, Yamagata, Bangalore A red and albino, and Lake Federsee albino stocks. Group 2 is composed of the New Delhi, Monterey, Stanford, Bangalore B, and Lake Stella stocks.

Figure 1 represents the combined streptomycin, penicillin and tetracycline results. There is no distinct clustering within Group 1. The Cameroun strain and the Yamagata strain show the highest degree of similarity (92%) of the ten stocks tested. The Bangalore A albino joins the first two at the 87% phenon level. Lake Federsee albino comes in at 83% and finally the Bangalore A red stock joins the group with 80% similarity.

Within Group 2 Monterey and Bangalore B strains show an 84% similarity, while Lake Stella and Stanford are 83% similar. These two subclusters join the New Delhi strain at the 80% phenon level to form Group 2 (Fig. 1).

[3] The mean division rate was determined for each stock in 0.1% Cerophyl solution by counting the number of organisms in a series of deep wells every 24 h.

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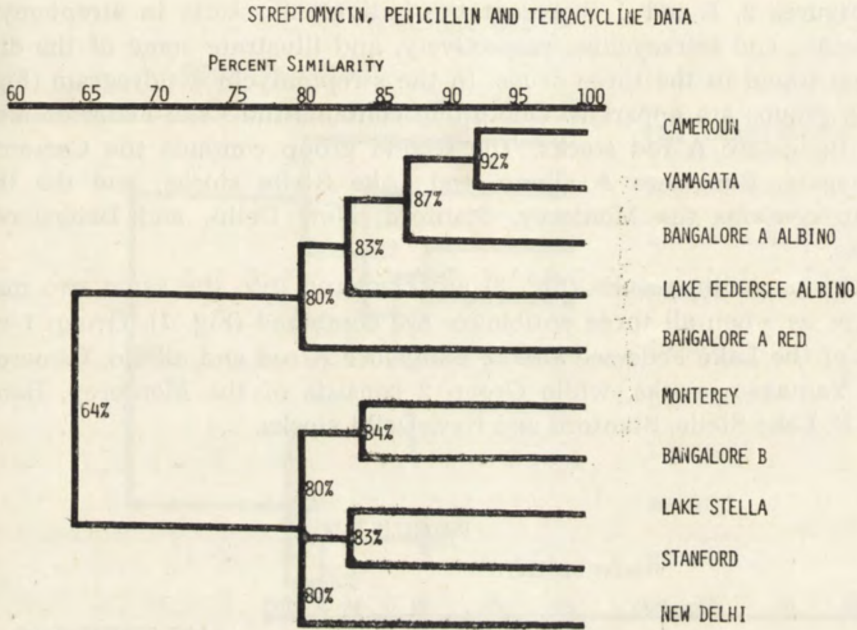

Fig. 1. Combined streptomycin, penicillin and tetracycline physiological data

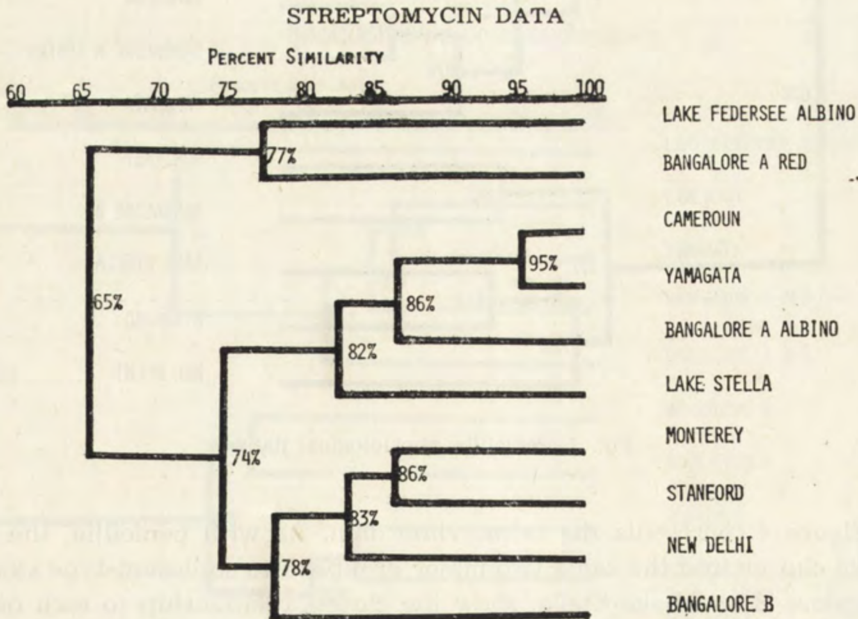

Fig. 2. Streptomycin physiological data

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Figures 2, 3 and 4 represent the individual results in streptomycin, penicillin and tetracycline, respectively, and illustrate some of the differences found in the three drugs. In the streptomycin dendrogram (Fig. 2) three groups are apparent. One group contains the Lake Federsee albino and Bangalore A red stocks. The second group contains the Cameroun, Yamagata, Bangalore A albino, and Lake Stella stocks, and the third group contains the Monterey, Stanford, New Delhi, and Bangalore B stocks.

The penicillin results (Fig. 3) are clustered into the same two major groups as when all three antibiotics are combined (Fig. 1). Group 1 consists of the Lake Federsee albino, Bangalore A red and albino, Cameroun, and Yamagata stocks, while Group 2 consists of the Monterey, Bangalore B, Lake Stella, Stanford and New Delhi stocks.

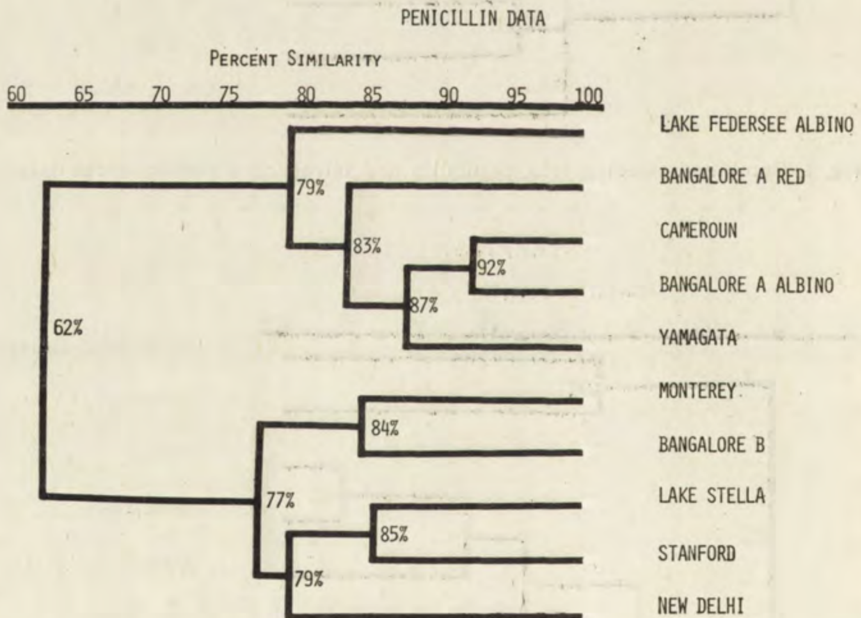

Fig. 3. Penicillin physiological data

Figure 4 represents the tetracycline data. As with penicillin, the ten stocks cluster into the same two major groups. The siniuosum-type stocks, Bangalore B and Lake Stella, show the closest relationship to each other (88%) in tetracycline as did the Bangalore A red and albino stocks (85%).

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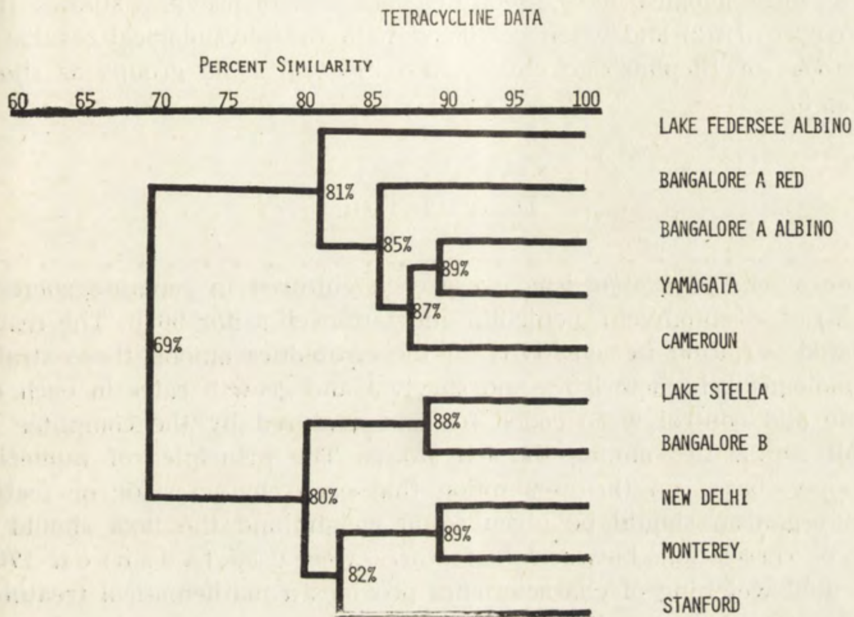

Fig. 4. Tetracycline physiological data

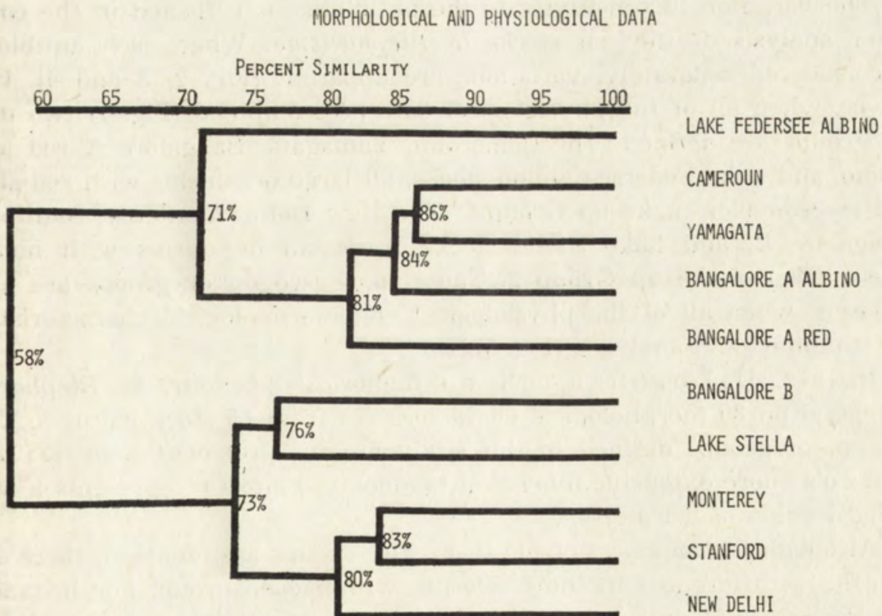

Fig. 5. Combined physiological and morphological data from the three antibiotics

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The morphological data directly correlates with previous studies (Di-Lorenzo 1972) and when combined with the physiological results the ten stocks of *Blepharisma* cluster into the two same groups as shown on Fig. 5.

Discussion

Ten stocks of *Blepharisma* were each cultured in separate solutions (10^{-5} M) of streptomycin, penicillin and tetracycline for 96 h. The results indicated variation in sensitivity to the antibiotics among these strains. Morphological characteristics and survival and growth rates in each antibiotic and control were coded for and analyzed by the computer for overall similarities among the ten stocks. The principles of numerical taxonomy, based on the assumption that each characteristic or feature of an organism should be given equal weight and the taxa should be based on correlations between the features, were used (Adanson 1757). The equal weighing of characteristics provides a mathematical treatment of characters and an estimation of resemblances, rather than key features (Sneath 1962). Cluster analysis in building taxonomic hierarchy is a major advance in the field of taxonomy (Sneath and Sokal 1963, 1973).

The variation in sensitivity to the antibiotics is reflected in the computer analysis of the ten stocks of *Blepharisma*. When each antibiotic is considered separately, variations are apparent (Fig. 2, 3 and 4). However, when all of the physiological data are combined (Fig. 1) two major groups are defined. The Cameroun, Yamagata, Bangalore A red and albino, and Lake Federsee albino stocks, all large organisms with rod-shaped macronuclei, make up Group 1. The New Delhi, Monterey, Stanford, Bangalore B, and Lake Stella stocks, all small organisms with noded macronuclei, make up Group 2. These same two major groups are also apparent when all of the physiological and morphological characteristics are combined and analyzed (Fig. 5).

In 1972, DiLorenzo devised a numerical taxonomy for *Blepharisma* based on 30 morphological characteristics from 45 stock cultures. The two major groups defined in this study are in agreement with DiLorenzo's more extensive numerical taxonomy. Figure 6 represents a modified version of her study.

Although the members of the two major groups are identical, there are significant differences in the clustering within each group. For instance, the Bangalore B and Lake Stella strains are members of *B. musculus*, one of the most controversial groups in *Blepharisma* taxonomy. Based

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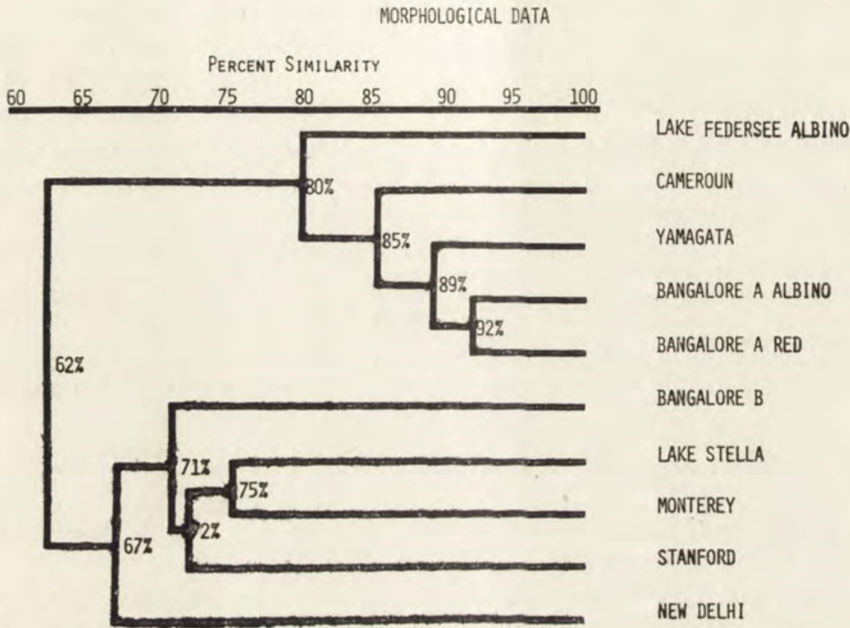

Fig. 6. Morphological data adapted from DiLorenzo's (1972) numerical taxonomic study

on the 71% similarity of the morphological characteristics. DiLorenzo separated these two sinuosum-like strains into separate species.

When the physiological data are considered, the two strains fall into two distinct subclusters of Group 2 (Fig. 1). Thus, the physiological results at first appeared to support the separation. However, physiologically the two strains have an 80% similarity which is 9% higher than their morphological similarity (Fig. 6). When both the physiological and morphological characteristics of these two strains are combined (Fig. 5), the 76% similarity is too high to consider them separate species. The addition of the physiological data does not justify separate species status for these two sinuosum types and would support them as varieties of *B. musculus* (Giese 1973).

Similarly, the combined morphological and physiological data (Fig. 5) show that the two *B. americanum* strains, Stanford and Monterey, have a stronger relationship (83%) than when either morphological (72%) or physiological (80%) characteristics are considered separately. This may indicate that the two California strains are more closely related than the previous studies indicate.

The physiological results also support the inclusion of the Bangalore

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A stocks, previously known as *B. intermedium*, with the japonicum group. Physiologically, the red and albino stocks have an 80% and 87% similarity, respectively (Fig. 1), with the other japonicum stocks studied. When the morphological characteristics are considered also, the four japonicum stocks, Bangalore A red and albino, Yamagata, and Cameroun, cluster at the 81% phenon level (Fig. 5) and therefore can be considered similar enough to belong to a single species, *B. japonicum*.

The difference between the red and albino stocks of the Bangalore A strain remains enigmatic. The albino mutant arose spontaneously in a clone culture of the Bangalore red strain and was believed to be morphologically "identical" to the red form, except for the lack of pigment. DiLorenzo's study (1972) showed that the red and albino organisms had a 92% similarity and could be considered different forms of the same organism. However, the performance of these two stocks in the three antibiotics is very different. The red always survives much better than the albino mutant (Table 1). The division rate of the control organisms is also considerably higher for the red stock (Table 2).

Physiologically there is only an 80% similarity between the two stocks (Fig. 1) as compared to the 92% morphological similarity found by DiLorenzo (Fig. 6). The physiological results imply that the difference between the red and albino forms of the Bangalore A strain could be more significant than the visible presence or absence of pigment.

The physiological characteristics presented here are but a few that can be studied for *Blepharisma*. It would be interesting to see how other properties, such as mating behavior, regeneration, photosensitivity, resistance to UV, etc., would fit into the numerical taxonomic scheme.

The results in this paper indicate that the addition of physiological data makes a valid contribution to the numerical taxonomy of *Blepharisma*. Although small differences between the morphological and physiological results occur when observing the levels of affinity at which the clusters join, the ten stocks cluster into the same two major groups as those found for morphological characteristics, and thus, agree with earlier studies (DiLorenzo 1972).

Controversies arising from morphological methods are made clearer when physiological data are considered. This paper supports the combining of the Bangalore A stocks with the japonicum group. However, it does not support the separation of the Lake Stella strain from the sinuosum group, and questions whether the difference between pigmentation and albinism in *Blepharisma* is as unimportant as previously indicated (Hirshfield et al. 1965).

In conclusion, the physiological differences found among the selected stocks of *Blepharisma* resulting from the effects of streptomycin, peni-

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cillin and tetracycline on survival and growth, provide additional information for the taxonomy of *Blepharisma*.

The computerized physiological results support the belief that numerical taxonomy is a useful and valid method for the classification of *Blepharisma* and encourage the use of additional non-morphological characteristics to emphasize further the similarities and differences among the strains. Numerical taxonomy and computer analysis are convenient and practical tools which should be seriously considered when studying the taxonomy of other genera.

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ZUSAMMENFASSUNG

Zehn Stammkulturen von *Blepharisma* wurden getrennt für 96 Stunden in Lösungen von Streptomycin, Penicillin und Tetracycline kultiviert. Die Überlebens- und Wachstumsrate für jede Stammkultur in jedem der Antibiotika wurde bestimmt. Nach 4 Tagen wurden die überlebenden Zellen fixiert, gefärbt und auf typische morphologische Merkmale hinuntersucht. Überlebens- und Wachstumsrate und charakteristische morphologische Merkmale wurden für die Methoden der numerischen Systematik codiert. Eine Computer-analyse wurde durchgeführt mit dem Ziel, allgemeine Ähnlichkeiten zwischen den Stammkulturen festzustellen. Die Stammkulturen fielen in Gruppen wenn morphologische und physiologische Daten analysiert wurden. Die physiologischen Daten gaben zusätzliche Information zur Systematik von *Blepharisma* und für die Klassifizierung kontroverser Arten, die in der Literature genannt werden.

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Studies on Some Hypermastigids (*Protozoa*) from the Termites of West Bengal, India

Synopsis. A systematic account of all the species of hypermastigids recorded so far from West Bengal, a state of Eastern India, consisting of nine species of *Holomastigotoides* Grassi and Foa, three species of *Spirotrichonympha* Grassi and Foa and two species of *Pseudotrichonympha* Grassi and Foa has been dealt with in this paper. This includes three new species, namely, *Holomastigotoides hollandei, H. emersoni* and *Spirotrichonympha roonwali*. In order to ascertain the taxonomic status, diagnostic characters and comparative mensural data have also been provided for some known species.

The aim of the present paper is to give a systematic account of all the species of three genera of hypermastigids, namely, *Holomastigotoides* Grassi and Foa, *Spirotrichonympha* Grassi and Foa and *Pseudotrichonympha* Grassi and Foa recorded from the gut of termites of West Bengal, a state of Eastern India. This is felt necessary because excepting some attempts by de Mello (1927, 1935) and Karandikar and Vittal (1954), that too on the hypermastigids from termites of Western India, no work of this type on this group of symbiotes of Indian termites has been undertaken so far. Over and above, the species described by de Mello (1927, 1935, 1942, 1950) consisting of 54% of the total species of hypermastigids recorded from Indian subcontinent have created many inconveniences as referred by Kirby on several occasions and Cross (1946), Honigberg (1970) and Emerson (1971). To quote Honigberg (1970), "many of his (de Mello's) description is rather inadequate and seems very likely that some organisms for which he proposed new names were assignable to known species".

In this context I have presented the morphology, various mensural

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data and characteristic features of each species recorded from West Bengal with a view to clearing up the confusions regarding the taxonomic status of some of the known species. In this connection mention may be made that barring a single species, *Spirotrichonympha pyriformis* Chakravarty and Banerjee, I have collected and studied all the species discussed here. The measurements are solely based on own material.

All the type-material will be deposited in the Zoological Survey of India.

Material and Methods

Termites were collected from different parts of West Bengal, India and from different habitats like timber depots, wood and logs, cemented brick-built wall, ply wood and pitch boards. The living flagellates were studied in fresh smears of gut contents diluted with 67% Locke's solution. For permanent preparation, thin and uniform smears of the gut contents mixed with very little quantity of 0.5% saline were drawn on slides, fixed in Schaudinn's fluid, Carnoy's fluid and Flemming's fluid and stained in Heidenhain's and Delafield's haematoxylin.

All the measurements were taken with the help of ocular micrometer and drawings were made with the aid of a camera lucida.

Hosts Examined

In connection with my taxonomic studies of termite flagellates from West Bengal I have examined the gut contents of four hosts, namely, *Heterotermes indicola* (Wasmann), *Coptotermes heimi* (Wasmann), *Cryptotermes havilandi* (Sjöstedt) and *Neotermes bosei* Snyder which are abundantly available in this state. In the gut contents of all these hosts flagellates are amazingly numerous sometimes predominating over the real food of their host in quantity. This is so, because there is a true symbiotic relationship between them. To quote Cleveland (1924), "the protozoa receive from termites food and lodging for which they give in return protozoan wood digestion products". However, among the four hosts mentioned above only two, namely, *Heterotermes indicola* and *Coptotermes heimi* harbour hypermastigids in large number particularly in the rectal region while the other two hosts contain only the polymastigids in their gut contents.

A list of termite hosts with their symbiotic hypermastigids is given in Table 1.

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Table 1

A List of Termite-Host with their Symbiotic Hypermastigids

Name of the host	No. of specimens examined	No. of specimens harbouring hypermastigids	Species of hypermastigid
Heterotermes indicola (Wasmann)	315	315	*H. hollandei* n. sp. *H. campanula* de Mello *H. sphaeroidalis* de Mello *H. globosus* de Mello *H. bengalensis* Chakravarty and Banerjee *H. magnus* Uttangi *P. indica* Chakravarty and Banerjee
Coptotermes heimi (Wasmann)	1000	1000	*H. emersoni* n. sp. *H. ogivalis* de Mello *H. rayi* Karandikar and Vittal *S. roonwali* n. sp. *S. froilanoi* Karandikar and Vittal *P. cardiformis* Karandikar and Vittal *P. indica* Chakravarty and Banerjee
Cryptotermes havilandi (Sjöstedt)	428	nil	nil
Neotermes bosei Snyder	72	nil	nil

Systematic Account

Holomastigotoides hollandei n. sp.

Fig. 1 Pl. I 1

Morphology

Body is more or less round in shape with finger like projection at the anterior end, sometimes bearing an apical pit. Flagella cover the whole of the body in dexiotropic rows. Of course, the posterior extremity of the body is thickly studded with much longer flagella. Nucleus is ovoidal in shape situated at the anterior part of the body. Axostyle is fibrillar and well developed reaching obliquely almost the posterior part of the body. Around the anterior end of the nucleus there is a dense homogeneous irregular mass, the prenuclear zone, which is very conspicuous in permanent preparations.

Measurements

	Mean	Range
Length of the body	71 μm	45.9–96 μm
Breadth of the body	51.4 μm	30.6–73.1 μm

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Length of the nucleus	13.3 μm	10.1–14.4 μm
Breadth of the nucleus	9.5 μm	6.8–11.9 μm
Length of flagella	12 μm	8.5–15 μm
Length of the flagella of posterior extremity	21.5 μm	17–25.5 μm
Ratio of body-length to body-width	1.4	1.2–1.7
Ratio of body-length to nuclear length	6.8	4.8–9.2

Type-Host: *Heterotermes indicola* (Wasmann);

Locality: Barrackpur (24-Parganas District), West Bengal, India.

Remarks

H. hollandei n. sp., by its round shape, moderately projected finger like elevation with apical pit at the anterior end, well developed oblique axostyle, distinct prenuclear zone and long flagella restricted to the posterior extremity has a distinct and separate identity and can be conveniently isolated from any other species of *Holomastigotoides* hitherto described. It, however, resembles *H. rayi* Karandikar and Vittal only having apical pit and distinct prenuclear zone. But *H. rayi* has oval shape, very large size, 200 × 125 μm (*H. rayi* obtained from the termites of West Bengal measures 104.1 × 66.2 μm) and faintly marked axostyle. Moreover, its posterior-fifth region is completely devoid of any dexiotropic flagellar rows which are replaced by some thickly grown irregularly arranged flagella.

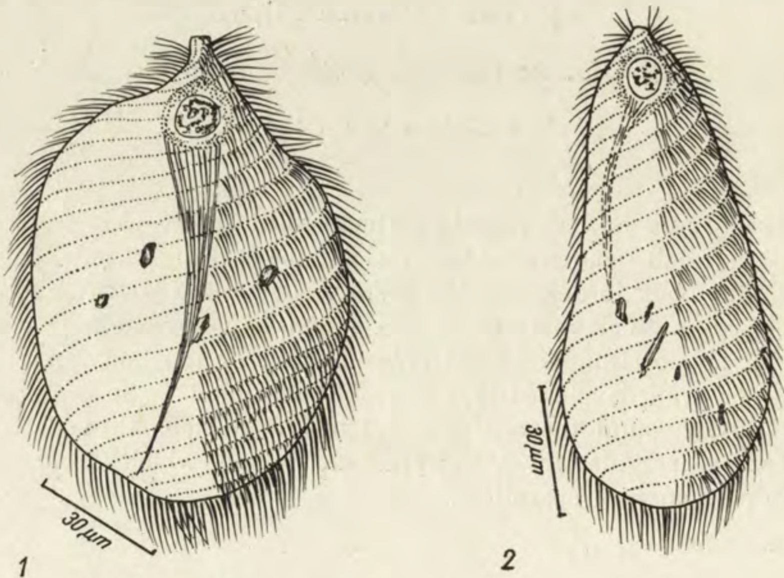

Fig. 1. *Holomastigotoides hollandei* n. sp. (arrangement of flagella partly shown)
Fig. 2. *Holomastigotoides emersoni* n. sp. (arrangement of flagella partly shown)

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Holomastigotoides emersoni n. sp.

Fig. 2 Pl. I 2

Morphology

It is club-shaped, the maximum width being near the posterior half of the body. There is a considerable elevation at the anterior end forming a nipple like structure while the posterior end is broadly round. Flagella are of two types. The shorter ones cover the major portion of the body dexiotropically and the longer ones are restricted only to the posterior extremity.

Nucleus is round in shape and located near the anterior pole of the body. Axostyle is ill developed. Around the anterior portion of the nucleus a dense and homogeneous prenuclear zone is distinctly visible in this species.

Measurements

	Mean	Range
Length of the body	97 µm	76.5–110.4 µm
Breadth of the body	40.5 µm	28.9–47.6 µm
Length of the nucleus	8 µm	6.8–10.2 µm
Breadth of the nucleus	7.5 µm	6.2–10.2 µm
Length of shorter flagella	7 µm	6–7.5 µm
Length of longer flagella	12 µm	10–15 µm
Ratio of body-length to body-width	2.2	1.9–2.5
Ratio of body-length to nuclear length	13.3	10.9–15

Type-host: *Coptotermes heimi* (Wasmann);
Locality: Falta (24-Parganas District), West Bengal, India

Remarks

H. emersoni n. sp. somewhat resembles *H. elongata* Uttangi as the latter possesses well developed prenuclear zone and lacks any axostyle (vs. ill developed axostyle of *H. emersoni*). But *H. elongata* differs considerably from the species under report in many major characters as presented in Table 2.

Holomastigotoides campanula de Mello, 1927

Fig. 3

Type-host: *Heterotermes* (= *Leucotermes*) *indicola* (Wasmann); Brancavara (Diu), India.

Additional hosts: *Heterotermes malabaricus* Snyder; Dharwar, India and *Coptotermes heimi* (Wasmann); Dharwar, India.

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Table 2

Comparison of *H. emersoni* n. sp. with Related Species

	H. elongata	*H. emersoni* n. sp.
Body configuration	'elongated, slender and cylindrical'	more or less club-shaped
Lenght (average)	167 μm	97 μm
Breadth (average)	50 μm	40.5 μm
Length-breadth ratio	3.3	2.2
Length of the nucleus	15 μm	8 μm
Breadth of the nucleus	10 μm	7.5 μm
Flagella	One type; 20 μm	two types; shorter ones – 7 μm longer ones – 12 μm
Axostyle	absent	ill developed

Morphology

Body resembles exactly a bell jar without apical knob. Flagella are of two types. Shorter ones are arranged all over the body in dexiotropic rows and the longer ones are restricted to the posterior extremity of the body. Nucleus is more or less oval and located near the anterior pole of the body. Axostyle is distinct but short. Prenuclear zone is absent.

Material: Collected from the gut of *Heterotermes indicola* (Wasmann); Locality: Indian Museum campus, Calcutta, West Bengal.

Measurements

In Table 3 the measurements of the specimens collected from the termites of West Bengal are given and compared with those of the specimens recorded by de Mello (1927) and by Karandikar and Vittal (1954).

Remarks

The specimens collected from the termites of West Bengal are by far the smallest in dimensions. But the average length-breadth ratio is much nearer to that of the specimens described by de Mello (1927). This species can be conveniently identified by its body-shape, axostyle and by the absence of prenuclear zone.

Holomastigotoides ogivalis de Mello, 1935

Fig. 4

Type-host: *Coptotermes* sp.; Daman, India.
Additional host: *Coptotermes heimi* (Wasmann); Dharwar, India.

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Table 3

Comparison of Measurements of *Holomastigotoides campanula* as Recorded by Different Authors

	Type specimens (based on 17 specimens)	Specimens as recorded by Karandikar and Vittal (No. of specimens not mentioned)	Specimens from West Bengal termites (based on 50 specimens)
Length of the body	70(37.5–118) μm	120 μm	52.6(42.5–69.1) μm
Breadth of the body	76(62–100) μm	100 μm	64.9(56.1–73.3) μm
Length of the nucleus	not given	not given	13.1 μm
Breadth of the nucleus	not given	not given	10.2 μm
Length of shorter flagella	not given	6–8 μm	5–7 μm
Length of longer flagella	not given	14–16 μm	9–10 μm
Ratio of body-length to body-width	0.9	1.2	0.8(0.7–0.9)
Ratio of body-length to nuclear length	–	–	4

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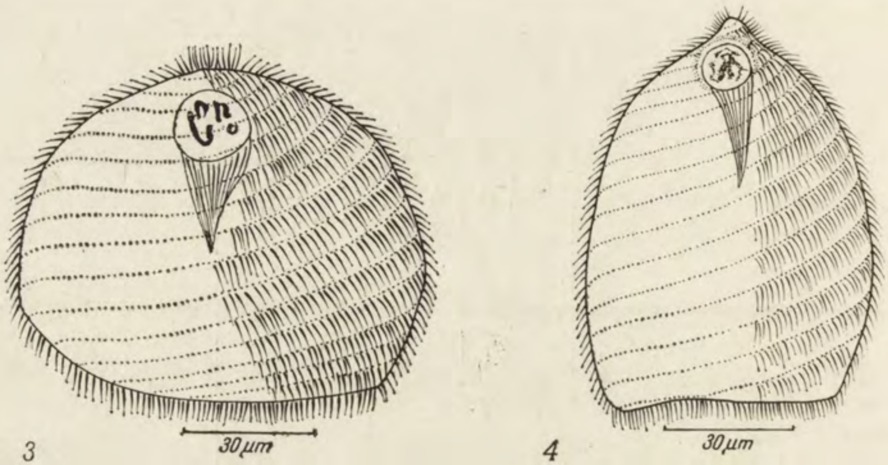

Fig. 3. ***Holomastigotoides campanula*** de Mello (arrangement of flagella partly shown)
Fig. 4. ***Holomastigotoides ogivalis*** de Mello (arrangement of flagella partly shown)

Morphology

Body shape resembles an inverted cup with a blunt finger like elevation at the anterior end. Shorter flagella occupy the whole of the body in dexiotropic rows leaving the posterior extremity which is thickly set with longer flagella. Nucleus is oval. Axostyle is distinct. Prenuclear zone is clearly visible in permanent preparation.

Material: Collected from the gut of *Coptotermes heimi* (Wasmann).

Locality: Haldia (Midnapur District) and Falta (24-Parganas District), West Bengal.

Measurements

	Mean	Range
Length of the body	82.3 μm	71.4–91.8 μm
Breadth of the body	55.7 μm	34–74.8 μm
Length of the nucleus	11.8 μm	10.2–12.7 μm
Breadth of the nucleus	8 μm	6.1–9.5 μm
Length of shorter flagella	—	6–7 μm
Length of longer flagella	—	9–10 μm
Ratio of body-length to body-width	1.5	1.2–1.6
Ratio of body-length to nuclear length	7.4	6.2–8.8

Remarks

De Mello (1935) described this species from *Coptotermes* sp. and noted the dimensions of only two specimens as 44 × 99 μm and 49 × 44 μm respectively. Subsequently Karandikar and Vittal (1954) recorded it from *Coptotermes heimi* (Wasmann) collected from

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Dharwar, India and put only the average dimensions of the species as 90 × 80 μm. *H. ogivalis* is, of course, readily recognizable by its conspicuous body shape, distinct axostyle and prenuclear zone.

Holomastigotoides sphaeroidalis de Mello, 1935

Fig. 5

Type-host: *Coptotermes* sp.; Daman, India.

Additional hosts: *Coptotermes heimi* (Wasmann); Dharwar, India and *Heterotermes malabaricus* Snyder; Dharwar, India.

Morphology

Body-shape is spherical as indicated by its specific name. Flagella are of one type covering the body dexiotropically. Nucleus is circular in shape and situated very near a point at the anterior end from which the flagellar bands seem to diverge out. Axostyle is well developed and prenuclear zone is distinct.

Material: Collected from the gut of *Heterotermes indicola* (Wasmann).

Locality: Indian Museum Campus, Calcutta, West Bengal.

Measurements

	Mean	Range
Diameter of the body	76.8 μm	67.5–86.2 μm
Diameter of the nucleus	7.8 μm	7.5–9.4 μm
Ratio of the diameter of the body to the diameter of the nucleus	9.9	9–11

Remarks

De Mello (1935) described *H. sphaeroidales* from *Coptotermes* sp. giving thereby the measurements of three specimens only as 41 × 39 μm, 43 × 41 μm and 49 × 47 μm respectively. Karandikar and Vittal (1954) recorded this species from *Coptotermes heimi* and *Heterotermes malabaricus* and described it as perfectly spherical with a diameter of 100 μm. My material from *Heterotermes indicola* which is a new host for this symbiote, has exactly the same shape as described by Karandikar and Vittal (1954) but possesses a distinct prenuclear zone which is not observed by any other previous worker.

Holomastigotoides globosus de Mello, 1935

Fig. 6

Type-host: indetermined; Brancavara Village (Diu), India.

Additional hosts: *Coptotermes* sp.; Daman, India and *Heterotermes malabaricus* Snyder; Dharwar, India.

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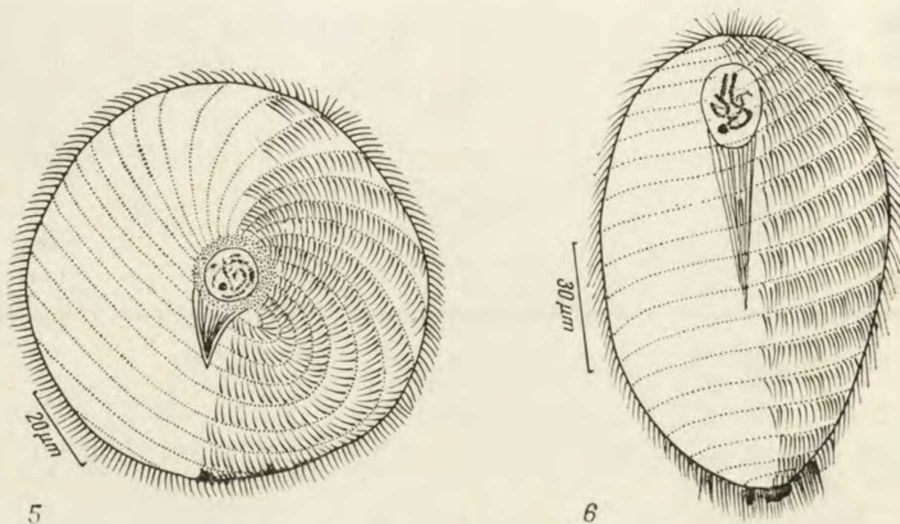

Fig. 5. ***Holomastigotoides sphaeroidalis*** de Mello (arrangement of flagella partly shown)
Fig. 6. ***Holomastigotoides globosus*** de Mello (arrangement of flagella partly shown)

Morphology

It is typically oval in shape. Flagella are of one type. Nucleus is ovoidal but rarely round and located near the anterior end of the body. Axostyle is moderately developed reaching almost the middle of the body. Prenuclear zone is lacking.

Material: Collected from the gut of *Heterotermes indicola* (Wasmann); Locality: Barrackpur (24-Parganas District), West Bengal.

Measurements

In Table 4 mensural data of the specimens collected from the termites of West Bengal is given and compared with that of the specimens recorded by earlier workers.

Remarks

H. globosus is a new record from the host *Heterotermes indicola*. The specimens from the termites of West Bengal differ from those of original description in being larger in dimensions and in having different length–breadth ratio. But their body-shape, nature of axostyle and absence of prenuclear zone are in conformity with those of *H. globosus*. Length-breadth ratio of the present material is, of course, nearer to that of the specimens recorded by Karandikar and Vittal (1954) from the host *Heterotermes malabaricus*.

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Table 4

Comparison of Measurement of *Holomastigotoides globosus* from Different Hosts

	by de Mello		by Karandikar and Vittal	Termites from West Bengal, *H. indicola*
	Coptotermes sp.	*Heterotermes* sp.	*H. malabaricus*	
Length of the body	69.4(35–100) μm	76.8(65–90) μm	140 μm	105.2(90.5–124.1) μm
Breadth of the body	54.4(35–80) μm	60.3(45–75) μm	90 μm	65.4(56.1–81.6) μm
Length of the nucleus	not given	not given	not given	12(10.5–15.3) μm
Breadth of the nucleus	not given	not given	not given	9.7(8.5–11) μm
Length of the flagella	not given	not given	8–10 μm	5–8 μm
Ratio of body-length to body-width	1.3	1.3	1.5	1.6(1.5–1.8) μm
Ratio of body-length to nuclear length	—	—	—	8.8(8.1–10.4)

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Holomastigotoides rayi Karandikar and Vittal, 1954

Fig. 7

Type-host: *Coptotermes heimi* (Wasmann); Dharwar, India.

Morphology

It is oval in shape. Apical pit is sometimes visible at the anterior end. Body is covered with two types of flagella. The shorter ones cover the body in a regular dexiotropic spiral while the longer ones are densely and irregularly set around the area of about one-fifth of the posterior region of the body. The latter are, of course, not steriocilia. Nucleus is ovoidal and anteriorly located. Axostyle is faintly visible. Prenuclear zone is distinct in stained preparation.

Material: Collected from the gut of *Coptotermes heimi* (Wasmann);

Locality: Haldia (Midnapur District) and Falta (24-Parganas District), West Bengal.

Measurements

Table 5

Comparison of Measurements of *Holomastigotoides rayi* with those of Type Specimens

	Specimens described by Karandikar and Vittal	Specimens from West Bengal termites
Length of the body	200 μm	104.1 (88.4–125.8) μm
Breadth of the body	125 μm	66.3 (61.2–70.5) μm
Length of the nucleus	not given	12.7 (11.9–13.6) μm
Breadth of the nucleus	not given	10.2 (9.3–11.5) μm
Length of flagella covering the major portion of the body	12–16 μm	10 μm
Length of flagella covering the posterior region of the body	nearly equal in length	14–15 μm
Area covered by the second type of flagella	about one-fifth of the body length	20.8 (18–23) μm (about one-fifth of the body length)
Ratio of body-length to body-width	1.6	1.6 (1.4–1.8)
Ratio of body-length to nuclear length	—	8.2 (7.8–9.3)

Remarks

H. rayi collected from the termites of West Bengal (same as the type host) is considerably smaller in dimension than that recorded by Karandikar and Vittal (1954) though length–breadth ratio of both the material is equal. Second type of flagella are longer than the first type in the specimens collected from West Bengal while Karandikar and Vittal (1954) described these as 'nearly equal in length'. The second type of flagella are irregular in its orientation. They cover approximately one fifth of the posterior region of the body as shown in Table 5.

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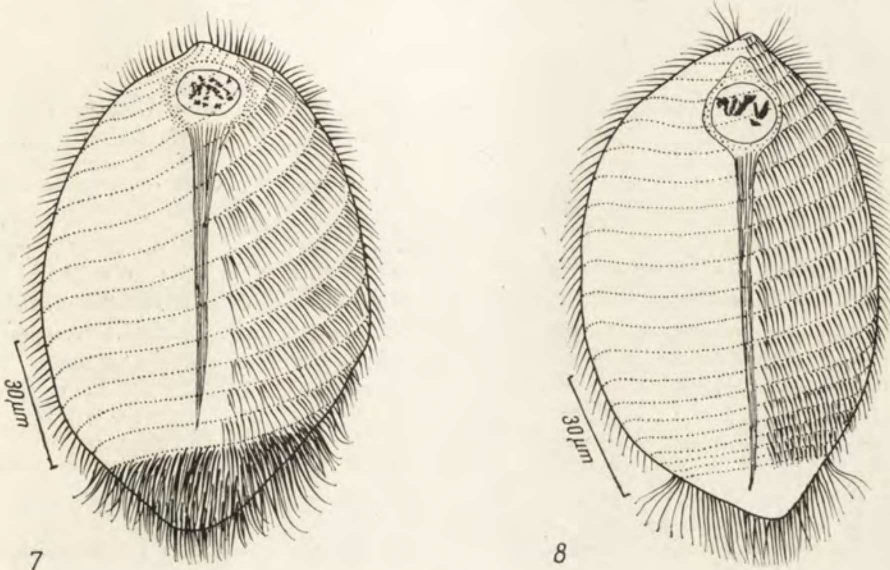

Fig. 7. ***Holomastigotoides rayi*** Karandikar and Vittal (arrangement of flagella partly shown)

Fig. 8. ***Holomastigotoides bengalensis*** Chakravarty and Banerjee (arrangement of flagella partly shown)

Holomastigotoides bengalensis Chakravarty and Banerjee, 1956

Fig. 8

Type-host: ***Heterotermes*** sp.; Calcutta, India.

Morphology

Body is more or less oval but sometimes elliptical. Anterior end is bluntly pointed resembling a nipple and the posterior end is rounded. Flagella are of two types. The shorter ones cover the major portion of the body in dexiotropic manner leaving a posterior portion which is glabrous and completely devoid of any flagellar band. The longer ones occupy the posteriormost portion of the body. Axostyle is well developed extending very near the posterior extremity of the body. Prenuclear zone is a densely granulated conical area around the anterior end of the nucleus.

Material: Collected from the gut of *Heterotermes indicola* (Wasmann); Locality: Indian Museum Campus, Calcutta, West Bengal.

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Measurements

Table 6

Comparison of Measurements of *Holomastigotoides bengalensis* from Different Hosts

	From type host	From *Heterotermes indicola*
Length of the body	81.9(27.8–135) μm	95.4(83.3–107.1) μm
Breadth of the body	53.2(21.4–87.5) μm	57(47.6–66.3) μm
Legnth of the nucleus	not given	12.9(12.7–13.6) μm
Breadth of the nucleus	not given	12.5(11.6–13.4) μm
Length of shorter flagella	not given	8–9 μm
Length of longer flagella	not given	11–12 μm
Ratio of body-length to body-width	1.5(1.2–1.8)	1.6(1.6–1.7)
Ratio of body-length to nuclear length	—	8.5(6.5–10.5)

Remarks

Chakravarty and Banerjee (1956) described *H. bengalensis* from an indetermined species of *Heterotermes* collected from various timber depots of Calcutta. I have recorded this species from the host *Heterotermes indicola* collected almost from the same locality. In course of cataloguing termite fauna occurring in West Bengal Maiti (1971) has stated that the host used by Chakravarty and Banerjee (1956) for termite flagellates is probably *Heterotermes indicola* because this is the only species of *Heterotermes* available in Calcutta.

The present material of *H. bengalensis* is quite identical with the types in all respect except that they are little larger in dimensions as presented in Table 6.

Holomastigotoides magnus Uttangi, 1962

Fig. 9

Type host: *Heterotermes indicola* (Wasmann); Gujarat and Mysore, India.

Morphology

It is large and ovoidal. Both the anterior and the posterior end is broadly round. Flagella are of one type covering the major portion of the body leaving a posterior glabrous region measuring about one fifth of the body length. This portion is completely devoid of any flagella. Nucleus is round and situated near the anterior end of the body. Axostyle is fibrous and moderately developed reaching beyond the middle region of the body. Prenuclear zone is clearly visible in stained preparation.

Material: Collected from the gut of *Heterotermes indicola* (Wasmann); Locality: Barrackpur (24-Parganas District), West Bengal.

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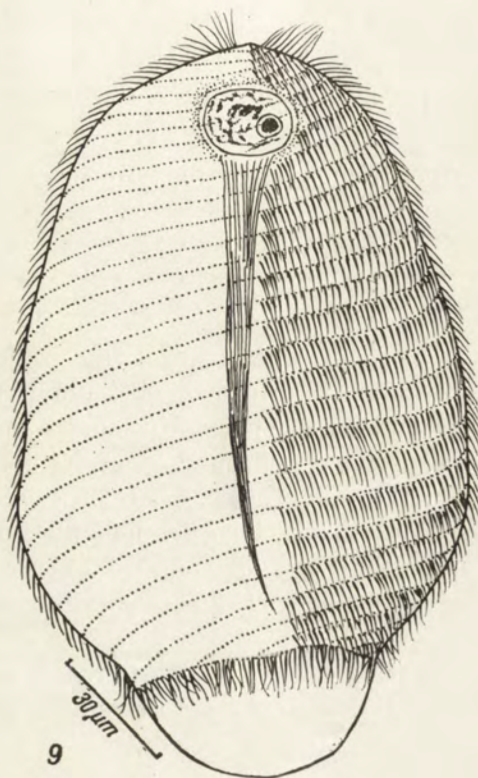

Fig. 9. ***Holomastigotoides magnus*** Uttangi (arrangement of flagella partly shown)

Measurements

Table 7

Comparison of Measurements of *Holomastigotoides magnus* from Different Hosts

	From type host	From the termites of West Bengal
Length of the body	165 µm	125.1(100.3–170) µm
Breadth of the body	110 µm	87.7(74.8–102) µm
Diameter of the nucleus	15–20 µm	15.3(13.6–17) µm
Length of flagella	not given	9–12 µm
Length of the glabrous portion of the body	one fifth of total body length	25.5 µm (one fifth of the total body-length)
Ratio of body-length to body-width	1.5	1.4(1.3–1.6)
Ratio of body-length to nuclear length	—	10.5(9–12.4)

Remarks

Uttangi (1962) described this species from the gut of *Heterotermes indicola* (Wasmann) collected from Gujarat and Mysore. The specimens

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collected from the termites of West Bengal (same as the type host) are quite identical with those described by Uttangi in their characteristic features except that they are smaller in dimensions.

Spirotrichonympha roonwali n. sp.

Fig. 10 P. I 3, 4

Morphology

Body is pyriform in shape with a finger like projection at the anterior end bearing a hyaline apical cap or operculum; the greatest width is near the posterior end of the body. Nucleus is more or less round. Prenuclear zone is dense and conical. Four spirally arranged flagellar bands arise from the base of centroblepharoplast which is situated at the tip of the body. These bands turned round the body dexiotropically and reach almost up to the posterior end. They contain numerous basal granules which give rise to flagella. Flagella are of one type measuring 13–16 μm in length.

Axostyle is thin consisting of many fine fibres. It extends beyond the middle region of the body but does not reach up to the posterior extremity.

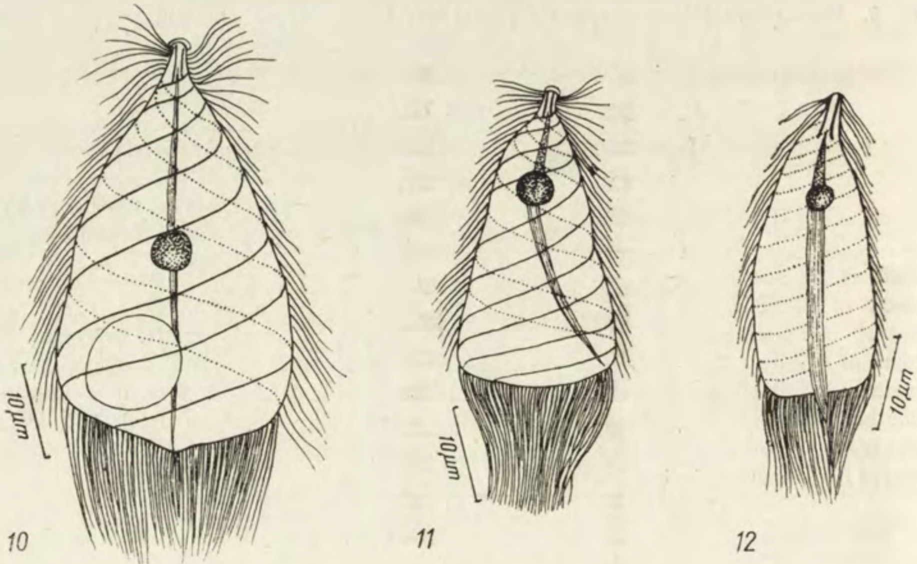

Fig. 10. *Spirotrichonympha roonwali* n. sp.
Fig. 11–12. *Spirotrichonympha froilanoi* Karandikar and Vittal

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Measurements

	Mean	Range
Length of the body	39 μm	35–42 μm
Breadth of the body	20.4 μm	18–22.4 μm
Length of the nucleus	5.1 μm	4.8–5.7 μm
Breadth of the nucleus	4.5 μm	4.2–5 μm
Length of the flagella	–	13–16 μm
Ratio of body-length to body-width	1–9	1.7–2.1
Ratio of body-length to nuclear length	7.6	7.5–8

Type host: *Coptotermes heimi* (Wasmann)
Locality: Falta (24-Parganas District), West Bengal, India.

Remarks

Being pyriform in body-shape and in having well developed beak like operculum at the anterior end, widened posterior part and an indistinct axostyle not extending up to the posterior end of the body, this symbiote is easily distinguishable from all the hitherto described species of *Spirotrichonympha* Grassi and Foa.

From the gut of *Coptotermes heimi* collected in Indian sub-continent four species of *Spirotrichonympha*, namely, *S. flagellata* Grassi and Foa, *S. elongata* Grassi, *S. froilanoi* Karandikar and Vittal and *S. karnataki* Karandikar and Vittal have been recorded so far. *S. roonwali* n. sp. distinctly differs from them because:

(1) *S. flagellata* has an elongated body rounded at the bottom and a thick and fibrous axostyle;

(2) *S. elongata* is elongated in body form, swollen at the middle and tappering at the ends with the axostyle extruded out from the posterior extremity of the body;

(3) *S. froilanoi* is conical in body-shape with broad and compressed posterior end containing conspicuously large flagella in a brush-like manner and the axostyle being distinct, thick and cord like; and

(4) *S. karnataki* has the same body shape as that of *S. froilanoi* but posterior extremity of the body is completely devoid of any flagella. This species has also a prominent axostyle projecting outside through the posterior surface.

Spirotrichonympha froilanoi Karandikar and Vittal, 1954

Fig. 11, 12

Type host: *Coptotermes heimi* (Wasmann); Dharwar, India. *Heterotermes malabaricus* Snyder; Dharwar, India.

Morphology

These flagellates have cone-shaped body with the anterior portion narrowed to a blunt end; posterior surface is broadest and usually de-

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pressed. Nucleus is somewhat round and located near the anterior third of the body. There are four flagellar bands originating from the centro-blepharoplast which is covered by hyaline apical operculim. Flagella are of two types. The shorter ones cover the entire body dexiotropically excepting the posterior surface which is studded with a bunch of very long flagella. They are so long in comparison to the body length and so thickly grown that they resemble a tuft of hair emerging out from a brush. Axostyle is very distinct and cord like in the post nuclear region. Usually it does not protrude outside but goes obliquely towards the posterior surface of the body. But in rare case it protrudes prominently to a shorter length through the posterior surface (Fig. 12).

Material: Collected from the gut of *Coptotermes heimi* (Wasmann);
Locality: Falta (24-Parganas, District), West Bengal.

Measurements

Table 8

Comparison of Measurements of *Spirotrichonympha froilanoi* with those from Original Description

	From original description	From the termites of West Bengal
Length of the body	50 μm	29(20.4–42.5) μm
Breadth of the body	25 μm	16.8(11.9–27.2) μm
Length of nucleus	–	4.5(3.4–5.1) μm
Breadth of the nucleus	–	4.2(3–4.6) μm
Length of first type of flagella	15 μm	9–10 μm
Length of second type of flagella	25 μm	15–20 μm
Ratio of body-length to body-width	2	1.7(1.4–2.1)
Ratio of body-length to nuclear length	–	6.4(5.2–8.5)

Remarks

Karandikar and Vittal (1954) described *S. froilanoi* from the gut of *Coptotermes heimi* and *Heterotermes malabaricus* collected in Dharwar (Mysore), India. They stressed on the character, namely 'presence of conspicuously long flagella spreading out from the posterior surface in a brush like manner' for specific diagnosis. This characteristic feature is non-exceptionally noted in the specimens collected from the termites of West Bengal. But as evident from Table 8, *S. froilanoi* from West Bengal is considerably smaller in dimensions and its length–breadth ratio deviates slightly from the previous record.

One notable feature observed by me in *S. froilanoi* is that the axostyle, though in rare cases, may protrude outside to a very short distance. This is not in conformity with the original description of the species.

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Karandikar and Vittal (1954) described another species, *S. karnataki* from *Coptotermes heimi* collected from the same place, Dharwar, and noted two characteristic features for its specific diagnosis; (i) axostyle always protrudes outside into a short or long distance and (ii) posterior surface of the body is completely devoid of any flagella. According to them, the first character is an 'outstanding feature which distinguishes this species from *S. froilanoi*'. But I have observed this in *S. froilanoi* obtained from the termites of West Bengal. Consequently, this can not be treated as the characteristic feature of *S. karnataki*.

So, the range of difference between the two species *S. froilanoi* and *S. karnataki* seems to be very narrow and their specific diagnosis is solely dependent on the presence or absence of flagella on the posterior extremity.

Spirotrichonympha pyriformis Chakravarty and Banerjee, 1956

Type host: ***Heterotermes*** sp; Calcuta, India.

Morphology

It is pear-sharped with the anterior end terminating to a point. The average dimension of the species is 30.02 × 15.48 μm and its length-breadth ratio varies from 1.25 to 2.50. Nucleus is spherical measuring 5.35 μm to 8.56 μm in diameter, situated at the anterior third of the body. It possesses an eccentric nucleolus and scattered chromatin granules. Axostyle is not detected.

Remarks

In course of present investigation I could not collect this species from the termites of West Bengal. The diagnostic characters and mensural data presented here are taken from original description (Chakravarty and Banerjee 1956).

Pseudotrichonympha cardiformis Karandikar and Vittal, 1954

Fig. 13

Type-host: ***Heterotermes malabaricus*** Snyder; Dharwar, India. ***Coptotermes heimi*** (Wasmann); Dharwar, India.

Morphology

Body is typically heart-shaped. Its anterior part consists of a bell like campanula which seems to be demarcated by a faintly stained circlet like line from the rest of the body. The central portion of the campanula is known as the rostral tube which has a constant length of 7.5 μm. The tube bears a more or less transparent hemispherical apical cap and is

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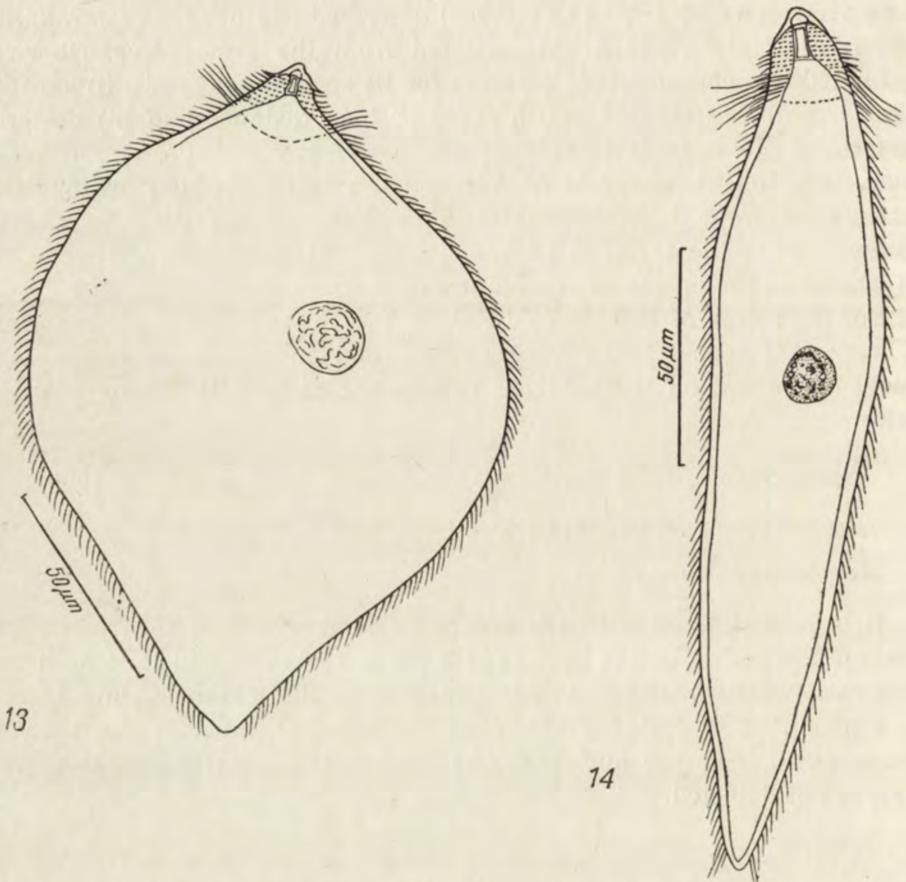

Fig. 13. ***Pseudotrichonympha cardiformis*** Karandikar and Vittal
Fig. 14. ***Pseudotrichonympha indica*** Chakravarty and Banerjee

closed at the bottom. The campanular surface is covered with three types of flagella distinguishable from one another in location and in length. First type of flagella is the shortest and immobile and are strictly confined to the rostral tube. Second type of flagella is the longest and arranged below the first one in a thick circular band. Third type of flagella is arranged in longitudinal rows in a leiotropic manner. They cover the campanula and the rest of the body except the short glabrous end of the posterior extremity. Nucleus is spherical and located generally above the middle region of the body. Parabasal apparatus is cord-like and generally found near the vicinity of the nucleus. Endoplasm is distinguishable into two zones, anterior one is finely granular while the posterior one contains large granules.

Material: Collected from the gut of *Coptotermes heimi* (Wasmann);
Locality: Falta (24-Parganas District); Haldia (Midnapur District), West Bengal.

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Measurements

Mensural data of the specimens collected from termites of West Bengal and that of the specimens described by Karandikar and Vittal (1954) are given in Table 9.

Table 9

Comparison of Mensural Data of *Pseudotrichonympha cardiformis* with that of the Specimens Described by Karandikar and Vittal (1954)

	From original description		From termites of West Bengal	
	Mean	Range	Mean	Range
Length of the body	–	100–125 μm	239.4 μm	213.7–352.5 μm
Breadth of the body	–	50–150 μm	131.2 μm	82.5–247.5 μm
Length of the nucleus	–	14 -27 μm	25.6 μm	22.5–37.5 μm
Breadth of the nucleus	–	14–27 μm	25.5 μm	20–33.7 μm
Ratio of body-length to body-width	–	–	1.9	1.3–2.6
Ratio of body-length to nuclear length	–	–	9.6	9.4–9.9
Length of rostrum	–	7–18 μm	7.5 μm	–
Length of campanula	–	28–35 μm (from *C. heimi*)	18.4 μm	16.8–22.5 μm
Campanula in maximum width	–	40–50 μm (from *C. heimi*)	40.6 μm	37.5–45 μm
Height of the apical cap	–	3–5 μm	3 μm	–
Length of first type of flagella	–	6–8 μm	–	5–7 μm
Length of second type of flagella	–	18–20 μm	–	17–18 μm
Length of third type of flagella	–	12–14 μm	–	9–10 μm

Remarks

P. cardiformis collected from termites of West Bengal is considerably bigger in dimension. But it is heart-shaped in appearance on the basis of which specific name *cardiformis* was assigned by Karandikar and Vittal (1954).

Pseudotrichonympha indica Chakravarty and Banerjee, 1956

Fig. 14

Type-host: *Heterotermes* sp.; Calcutta, West Bengal.

Morphology

It is much elongated with its broadest part being almost near the middle. Campanular region is very short in comparison to body-length. Rostral tube has the length of 6 to 6.5 μm bearing the apical cap. Flagella are of three types occupying the same position as described in *P. indica*. Nucleus is more or less circular and located near the middle of

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the body containing scattered chromatin granules. But sometimes, though very rarely, it is found to occupy the posterior region. Endoplasm is granular sometimes containing fragment of wood.

Material: Collected from the gut of *Coptotermes heimi* (Wasmann);

Locality: Falta (24-Parganas District); and from *Heterotermes indicola* (Wasmann); locality: Indian Museum campus, Calcutta, West Bengal.

Measurements

Table 10

Comparison of Mensural Data of *Pseudotrichonympha indica* with that of the Specimens Described by Chakravarty and Banerjee (1956)

	From original description		From the termites of West Bengal	
	Mean	Range	Mean	Range
Length of the body	—	220–300 μm	193.8 μm	165–221.2 μm
Breadth of the body	—	38–55 μm	38.2 μm	22.5–52.5 μm
Length of the nucleus	—	10.26–11.4 μm	15.1 μm	10.2–18.7 μm
Breadth of the nucleus	—	10.26–11.4 μm	13.5 μm	10.2–15 μm
Ratio of body-length to body-width	—	—	5	4.2–7.3
Ratio of body-length to nuclear length	—	—	13.5	11.5–15.9
Length of rostrum	—	—	—	6–6.5
Length of campanula	—	—	16.6 μm	16.3–17 μm
Campanula in maximum width	—	—	12 μm	9.3–14 μm
Height of the apical cap	—	—	3.2 μm	—
Length of first type of flagella	—	—	—	5–6 μm
Length of second type of flagella	—	—	—	11–12 μm
Length of third type of flagella	—	—	—	7–8 μm

Remarks

Chakravarty and Banerjee (1956) described *P. indica* from some unidentified species of *Heterotermes*. I have recorded it from the gut of two hosts as mentioned above. The specimens recorded from *Heterotermes indicola* are exactly identical with the types in all characteristic features except that they are smaller in dimensions.

As stated earlier the host used by Chakravarty and Banerjee (1956) being *Heterotermes indicola*, *Coptotermes heimi* is a new host recorded for *P. indica*.

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RÉSUMÉ

Le travail passe en révue systématique toutes les espèces des *Hypermastigida* trouvées jusqu'à présent au Bengal Occidental (un état dans la partie est des Indes), à savoir neuf espèces de Holomastigoides Grassi et Foa, trois espèces de *Spirotrichonympha* Grassi et Foa, et deux espèces de *Pseudotrichonympha* Grassi et Foa. Trois espèces nouvelles se trouvent dans ce nombre, notamment: *Holomastogoides hollandei, H. emersoni* et *Spirotrichonympha roonwali*. Pour mettre en évidence leur position taxonomique, les caractères diagnostiques et les données métriques comparatives sont fournies également pour quelques espèces déjà connues.

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EXPLANATION OF PLATE I

1: *Holomastigotoides hollandei* n. sp. × 410
2: *Holomastigotoides emersoni* n. sp. × 325
3–4: *Spirotrichonympha roonwali* n. sp. × 957

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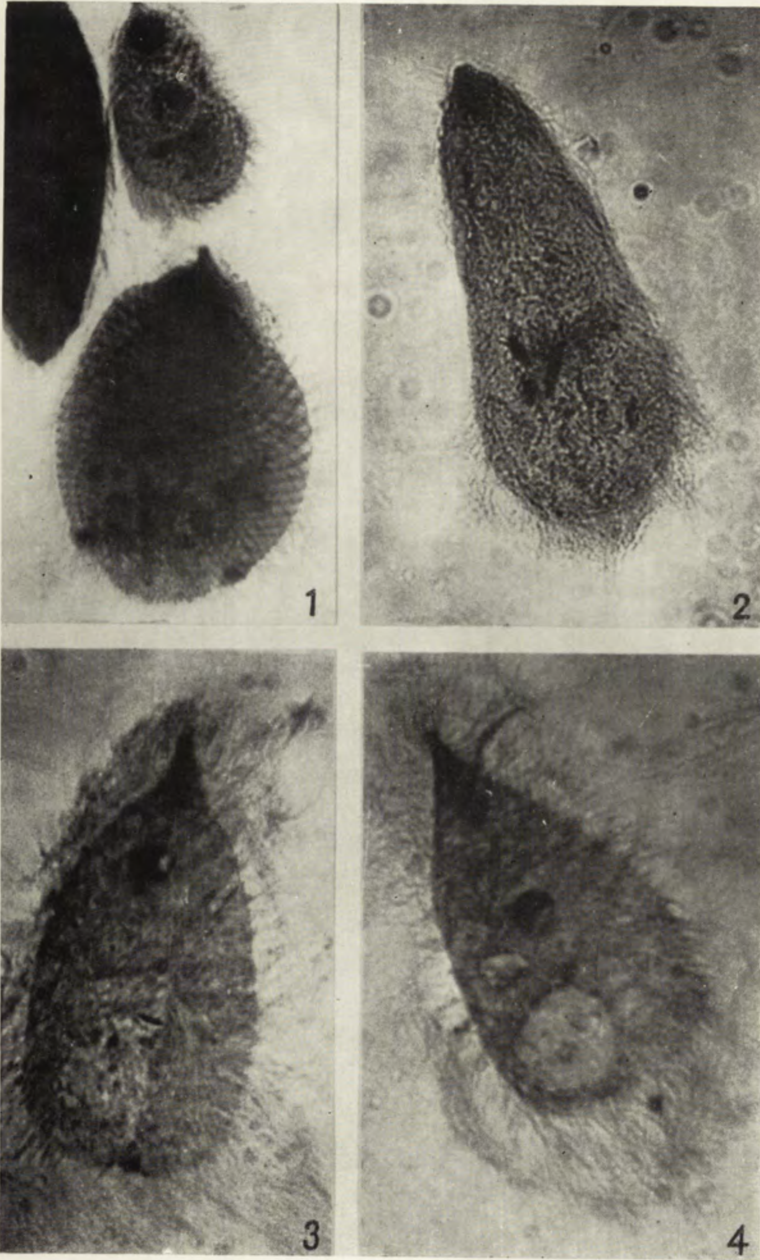

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Three Species of Flagellates of the Genus *Retortamonas* (*Mastigophora* : *Retortamonadidae*) from *Gryllotalpa africana* in India

Synopsis. The morphology of two new species of the genus *Retortamonas*, besides *R. wenrichi* Stabler, 1944 is described from the gut contents of *Gryllotalpa africana*. The presence of a knobbed rod-like structure behind the nucleus is recorded from *R. wenrichi*. *R. aurangabadensis* n. sp. is characterized by a sac-like body and a long tapering tail, a blepharoplastic complex of three granules lateral to the nucleus, a cytostome bordered by three fibrils and a deeply staining cap-like body at the anterior end. *R. nagabhushani* n. sp. is characterized by a spindle shaped body devoid of a posterior tail, a pair of blepharoplasts and a single cytostomal fibril.

Flagellates belonging to this genus were first recorded and named by Grassi (1879) on the basis of material collected from the mole cricket. He named it *Retortamonas gryllotalpae*, with a very brief description. Later in 1881, he gave a longer description of these forms together with illustrations, but placed them in the genus *Plagiomonas*, without assigning any specific reasons. Doflein (1901) referred this species to the genus *Bodo*, while Stiles (1902) reviewed Grassi's work and suggested that *Plagiomonas* must be regarded as a synonym of *Retortamonas*. Mackinnon (1911) described a flagellate from the caddis fly larva, naming it *Embadomonas agilis*. Later in 1912, she recorded this species as

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well as a second species *E. alexeieffi* from crane fly larvae. Subsequently several species of this genus were described by different workers. Wenrich (1932) made a detailed study of similar flagellates from mole cricket and in view of their similarity to *R. gryllotalpae*, synonymized *Embadomonas* with *Retortamonas*, and named a new family *Retortamonadidae* to accommodate these flagellates. Though some workers like Bishop (1934) and Dobell (1935) disagreed with Wenrich's conclusions, most of the recent workers have followed the latter's course and placed these flagellates in the genus *Retortamonas*.

Species of this genus inhabit the gut of several arthropods and as many as 11 species have been described so far. During the course of a survey of the flagellate fauna of the mole cricket *Gryllotalpa africana* in India, carried out by the author, three species of this genus were encountered. On detailed examination, one of them was found to resemble *R. wenrichi* Stabler, 1944 while the other two were different from all the recorded species. The present communication contains a detailed descriptions of these forms and a general comparison of the species of this genus from various arthropods.

Material and Methods

During the present survey, three species of the genus *Retortamonas* were observed in the contents of the hind gut of *Gryllotalpa africana*. Two of these, which are new to science, were found in abundance during the months of July and August. Their incidence was relatively very low during the rest of the year. The incidence of the third species, *R. wenrichi* Stabler, 1944 was more frequent, being found throughout the year.

The parasites were examined in the living condition with the help of vital stains such as methylene blue. *R. wenrichi* and *R. aurangabadensis* n. sp. were conspicuous in the living condition because of their large size and typical shape. The permanent preparations were stained with Giemsa's stain after exposure to osmic vapours and fixation in methanol. For the wet preparations, the smears were fixed in Schaudinn's fluid and stained with Phospho-tungstic haematoxylin. The drawings were made with the help of a camera lucida at a magnification of 2000 ×.

Results

Retortamonas wenrichi Stabler, 1944

(Fig. 1)

Morphology

The parasite shows the anterior end rounded, the middle region much bulged and the posterior region tapering into a tail-like projection. The projection is variable in length, showing a range of 5.1–57.1 μm, with an

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average of 20.6 μm. It is not uniform in its thickness. In some it is long and filamentous (Fig. 1 *5*, *7*), but in the majority of forms it is tubular and tapering (Fig. 1 *1–4*, *8*) and always apparently flexible.

A pair of flagella arise from two blepharoplasts situated at the anterior end of the organism (Fig. 1 *2*). Both the flagella are of the same length and thickness and in some of the organisms either one or both the flagella end in acronemes of variable length (Fig. 1, *1*, *3*, *5*).

The cytostome is large and conspicuous and extends up to one-third of the body length (Fig. 1 *3*, *4*, *10*). The edge of the cytostome is bordered on one side by a distinctly staining cytostomal fibril. The fibril extends along the outer border of the cytostome and recurves posteriorly (Fig. 1 *1*, *3*). In some forms another thin and delicate fibril was also seen running parallel to the first (Fig. 1 *1*, *4*).

The nucleus is situated behind the blepharoplasts. It is ovoidal (Fig. 1 *2*, *7*), spherical (Fig. 1 *6*, *8–10*) or irregular in outline (Fig. 1 *1*, *3*, *4*) and has uniformly distributed chromatin granules (Fig. 1 *10*) or a central endosome surrounded by a clear space (Fig. 1 *9*).

In some of the flagellates a peculiar structure was also observed behind the nucleus. It is a short or somewhat longer rod-like structure extending backwards from the nuclear membrane and ending in a swollen knob-like head (Fig. 1 *4*, *5*). The significance of this structure is not known.

Measurements (range and average within brackets)

Length of the body	13.9–24.7 μm	(19.2 μm);
Width of the body	7.7–15.9 μm	(11.3 μm);
Length of the spike	5.1–57.1 μm	(20.6 μm);
Length of the nucleus	1.5–5.1 μm	(3.5 μm);
Width of the nucleus	2.6–6.2 μm	(3.7 μm);
Length of the flagellum I	9.3–29.8 μm	(21.2 μm);
Length of the flagellum II	10.8–28.8 μm	(21.4 μm);
Length of the cystome	5.7–8.2 μm	(7.7 μm);
Width of the cystome	3.1–7.2 μm	(4.8 μm);

Comment

This species was first described by Stabler (1944) from the mole cricket *Gryllotalpa hexadactyla*. It was later recorded by Bhaskar Rao (1968) from *G. africana* in India. The parasite described above is also from the same host (i.e., *G. africana*), but shows some minor variations.

The variations in the body dimensions of the three forms is shown in Table 1.

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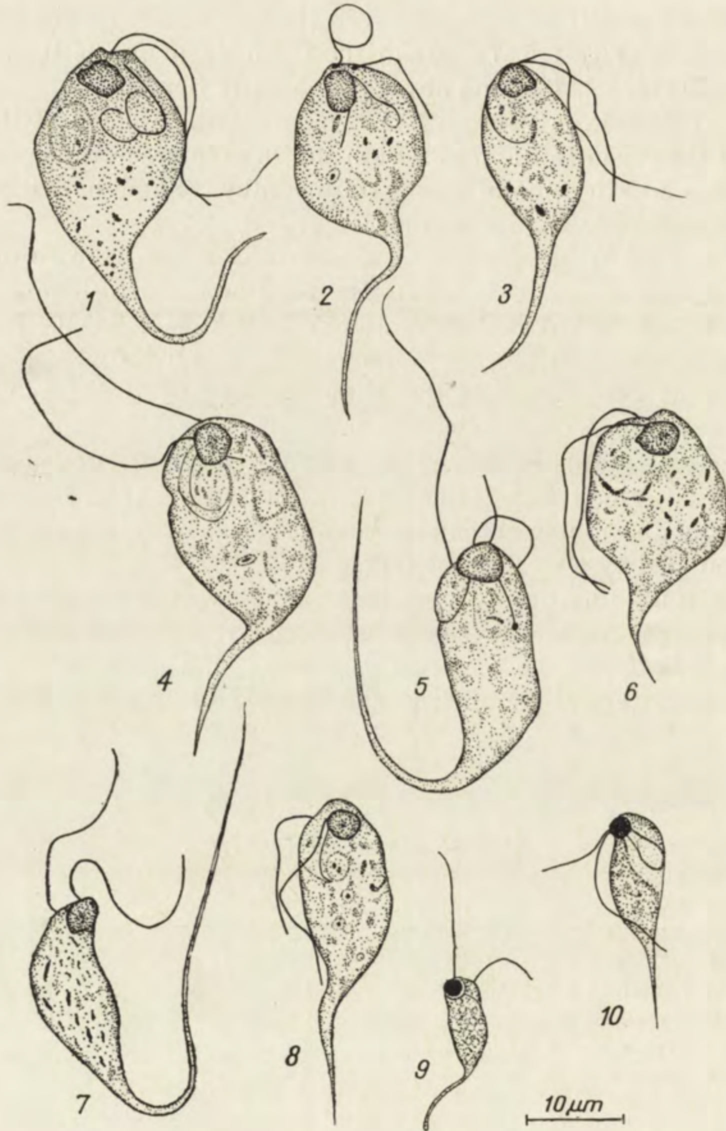

Fig. 1. *Retortamonas wenrichi* Stabler, 1944 (Figs. *1-8* from smears exposed to osmic vapours, fixed in methanol and stained with Giemsa stain, Figs. *9, 10* from smears fixed in Schaudinn's fluid and stained with phosphotungstic haematoxylin), *1* — Showing a tubular tail, acronematic flagellum and two cytostomal fibrils, *2* — Showing two blepharoplasts, *3, 6, 8* — Showing general structure, *4* — Showing a large cytostome with two fibrils and short rod-like body behind nucleus, *5* — Showing a long rod-like body, acronematic flagellum and filamentous tail, *7* — Showing a long filamentous tail, *9* — Showing a large endosome in nucleus, *10* — Showing the cytostome and the nucleus filled with chromatin granules

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Table 1

Variations in the Body Dimensions of *Retortamonas wenrichi* Stabler, 1944
(All measurements in microns)

Sr. No.	Author	Size of the body proper	Length of the body with the flagella
1	Stabler, 1944	12.2–19.0×3.8–8.4 (16.0×5.9)	3.1–58.5 (23.2)
2	Bhaskar Rao, 1968	8.0–25.0×4.0–15.0 (18.0×8.2)	2.0–38.5 (21.3)
3	Present author	13.9–24.7×7.7–15.9 (19.2×11.3)	5.1–57.1 (20.6)

It is evident from the Table that the organism described here is almost twice as broad as that described by Stabler (1944) and also slightly longer.

The flagella are almost equal in all the forms, but they are about as long as the body proper in this form, as against longer flagella (one-and-a-half to two times the body length) described by Stabler (1944). The only other major difference in this form is the presence of the rod-like structure with a knob-like head just behind the nucleus.

Species: *Retortamonas wenrichi* Stabler, 1944
Host: *Gryllotalpa africana*
Habitat: Hindgut
Locality: Aurangabad, Maharashtra, India.

Retortamonas aurangabadensis n. sp.

(Fig. 2)

Morphology

The parasites are characteristic, having a large and bulging body proper and a very long and narrow tapering spike posteriorly. The body proper is elongated, ovoidal (Fig. 2 *3, 4, 5*) or spindle shaped (Fig. 2 *2, 5, 10*). The anterior end is bluntly rounded (Fig. 2 *4, 9, 10*) or somewhat conical and projected into a beak-like structure (Fig. 2 *1, 5, 11*). The

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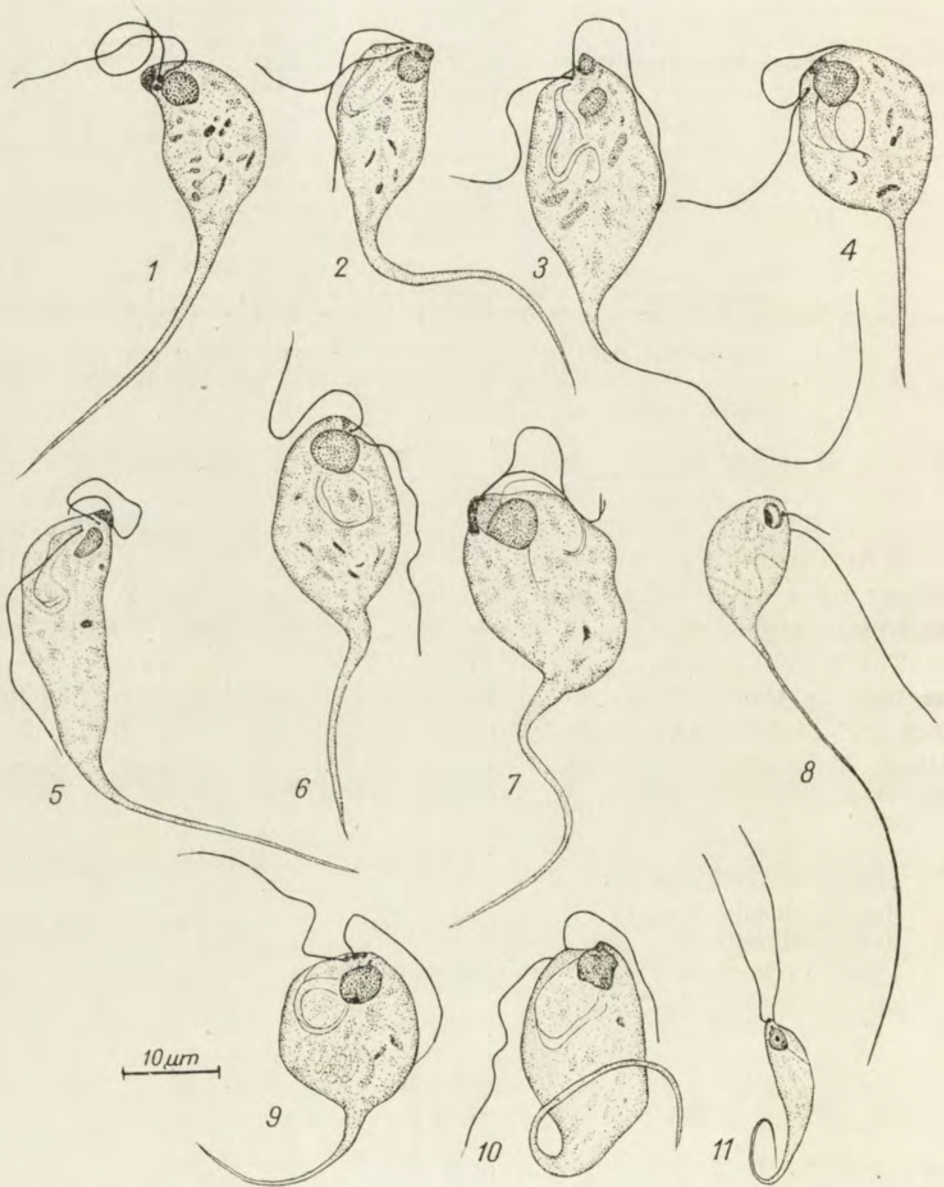

Fig. 2. *Retortamonas aurangabadensis* n. sp. (Figs. *1–7*, *9*, *10* from smears exposed to osmic vapours, fixed in methanol and stained with Giemsa stain. Figs. *8*, *11* from smears fixed in Schaudinn's fluid and stained with Phosphotungstic haematoxylin), *1* — Showing conical anterior end, with cap-like body and acronematic flagellum *2*, *4*, *6*, *10* — Showing general structure, *3*, *5* — Showing three basal granules and the origin of the flagella and fibrils, *7* — Showing a large and diffused cap-like body, *8* — Showing a long filamentous tail and nucleus with a large endosomal plaque in one half, *9* — Rounded form showing fibrils and acronematic flagella, *11* — Showing the nucleus with central endosome and chromatin granules

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middle of the body is relatively large and bulging while the posterior part is conical and suddenly tapers into the long tail-like spike.

The blepharoplastic complex is situated just below the anterior end. In well differentiated specimens, it seems to comprise of three granules, two giving rise to the two flagella and the third to the cytostomal fibrils (Fig. 2 *3, 5*). The granules are placed slightly towards one side (Fig. 2 *2, 3, 5*). The two flagella are almost equal in length, being somewhat longer than the body proper and end in acronemes in some cases (Fig. 2 *1, 3, 5, 6, 9*). The cytostome is distinct and relatively large, extending upto the middle of the body proper in many cases. The edges of the cytostome are bordered by three chromophilic fibrils. Of the three fibrils, two run close together along the outer border of the cytostome upto the posterior end of the pouch and then recurve anteriorly (Fig. 2 *3, 5, 7*). In some cases these fibrils appear to be coiled (Fig. 2 *4, 9*). The third cytostomal fibril appears to be relatively very fine and delicate and could be seen only in some well stained preparations. It arises along with the other two fibrils, but runs inwards, along the inner border of the oral pouch (Fig. 2 *3*). The inner fibril is also much shorter than the two outer fibrils.

The nucleus is just behind the anterior end of the body. It is spherical or ovoidal, and has a small central endosome surrounded by chromatin granules (Fig. 2 *11*) or a very large plaque lying against the membrane in one half (Fig. 2 *8*).

The pellicle is relatively well developed, giving the body a rigid shape. The cytoplasm contains a variable number of deeply staining granules and bacteria.

A very characteristic feature of the organism is the presence of a darkly staining body of variable size situated at the extreme anterior end of the body. It is relatively large and either spherical (Fig. 2 *2, 3*) or hemispherical (Fig. 2 *1, 5*), forming a cap-like structure. In some forms (Fig. 2 *7, 9*) it stains less intensely and appears to be diffuse.

Measurements (range and average within brackets)

Length of the body	15.4–25.7 μm	(20.7 μm);
Max. width of the body	8.2–17.0 μm	(12.1 μm);
Length of the spike	4.6–60.1 μm	(27.7 μm);
Length of the nucleus	1.5–5.7 μm	(3.8 μm);
Width of the nucleus	2.1–6.2 μm	(4.3 μm);
Length of the flagellum I	11.3–31.4 μm	(23.0 μm);
Length of the flagellum II	10.3–32.4 μm	(23.3 μm);

Discussion

From the available literature, it is seen that eleven species of the genus *Retortamonas* have been described so far from insects. These are:

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(1) *R. gryllotalpae* Grassi, 1879
(2) *R. agilis* Mackinnon, 1911
(3) *R. alexeieffi* Mackinnon, 1912
(4) *R. belostomae* Brug, 1922
(5) *R. blattae* (Bishop, 1931)
(6) *R. phyllophagae* (Travis and Becker, 1931)
(7) *R. termitis* Kirby, 1932
(8) *R. caudacus* Geiman, 1932
(9) *R. wenrichi* Stabler, 1944
(10) *R. pericopti* Laird, 1956
(11) *R. toddi* Bhaskar Rao, 1968

Of all these species only *R. wenrichi* has a characteristically long tail. The organism just described also has a similar posterior tail and comes close to *R. wenrichi* in its body dimensions. However, there are several features distinguishing the two species.

The blepharoplastic complex here consists of three distinct granules placed somewhat lateral to the nucleus, while *R. wenrichi* has two granules at the anterior end. The borders of the cytostome in *R. wenrichi* have two fibrils whereas this organism has a larger cytostome bordered by three fibrils, two along the outer edge and a shorter and finer third fibril towards the inner border. The nucleus of *R. wenrichi* has diffused chromatin or a large endosome in rare cases. But this parasite has either a small central endosome surrounded by chromatin granules or a very large plaque lying againts the membrane in one half. Besides these differences this organism has a deeply staining cap-like body of variable size at the extreme anterior end of the body. Though the significance of this body is not clear, such a structure has never been reported in any of the species described so far.

In view of these differences the organism is considered new to science and named *Retortamonas aurangabadensis* n. sp., after the locality where it was found.

Species: *Retortamonas aurangabadensis* n. sp.
Host: *Gryllotalpa africana*
Habitat: Midgut
Locality: Aurangabad, Maharashtra, India.

Retortamonas nagabhushani n. sp.

(Fig. 3)

Morphology:

The organism is somewhat elongated and spindle shaped, with the maximum diameter just behind the middle of the body. The anterior end

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is somewhat rounded (Fig. 3 *4, 7, 8, 10*) or conical (Fig. 3 *1, 5, 6, 11*) while the posterior end is more tapering and ends in a pointed tip (Fig. 3 *1, 2, 5*). In rare cases the posterior end is drawn out into a spine-like structure of variable length (Fig. 3 *6*).

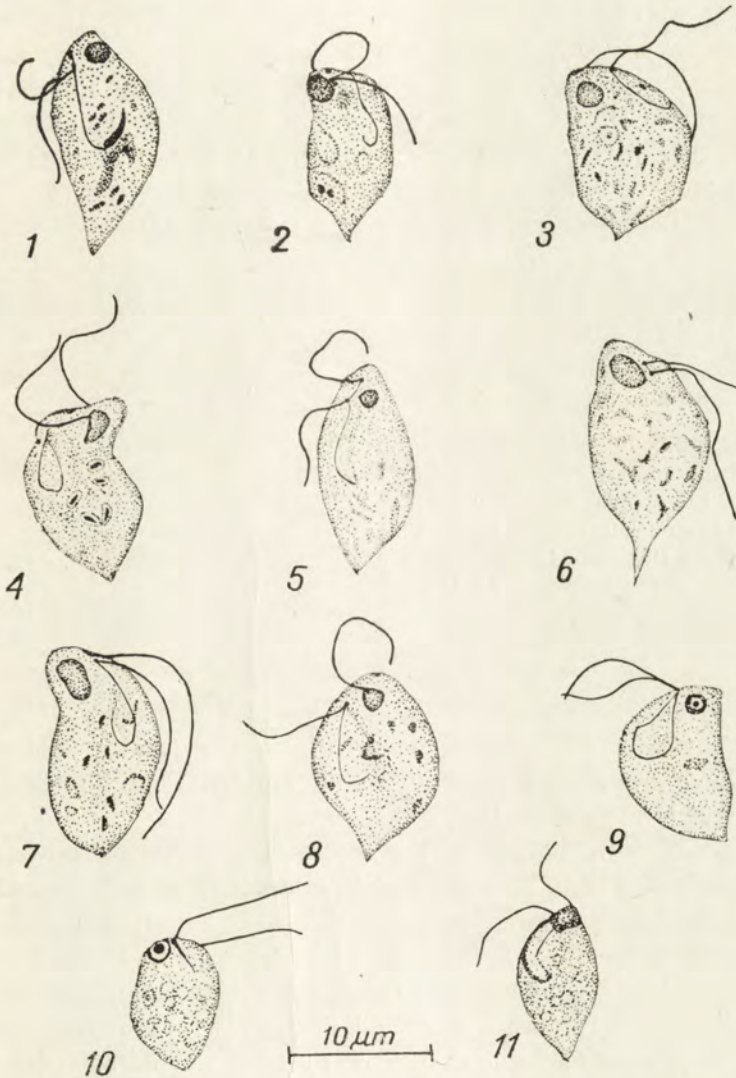

Fig. 3. ***Retortamonas nagabhushani*** n. sp. (Figs. *1–8* from smears exposed to osmic vapours, fixed in methanol and stained with Giemsa stain. Figs. *9–11* from smears fixed in Schaudinn's fluid and stained with Phosphotungstic haematoxylin) *1* — Spindle shaped form showing pointed posterior end, two blepharoplasts and cytostomal fibril, *2, 7* — Showing the origin of the cytostomal fibril, *3, 4, 8, 11* — Showing general structure, *5* — Showing two blepharosplasts, rhizoplast and origin of flagella, *6* — Showing rhizoplast and spine-like posterior end, *9, 10* — Showing endosome in nucleus

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There are a pair of blepharoplasts situated a little behind the anterior end, by the side of the nucleus (Fig. 3 *1, 5, 6*). The granules are small, situated one above the other, about 1 µm apart, and connected by a delicate rhizoplast (Fig. 3 *5, 6*). The upper granule gives rise to a single flagellum while the lower one is the point of origin of the other flagellum as well as a cytostomal fibril (Fig. 3 *1, 2, 5, 8*). The two flagella are subequal and slightly shorter than the body.

A narrow sac-like cytostome is present, extending almost up to the middle of the body, on the same side where the blepharoplasts are situated. The outer border of the pouch has a well defined cytostomal fibril, which extends up to the posterior end and recurves anteriorly around the base of the oral pouch (Fig. 3 *1, 2, 7, 8, 11*).

The nucleus, lying just below the anterior end, is small and has a central endosome and a ring of peripheral chromatin granules (Fig. 3 *9, 10*). The cytoplasm has several deeply staining granules and bacteria.

Measurements (range and average within brackets)

Length of the body	9.8–18.0 µm	(13.7 µm);
Width of the body	6.2–9.3 µm	(7.6 µm);
Length of the nucleus	1.0–3.6 µm	(2.0 µm);
Width of the nucleus	1.0–4.6 µm	(2.0 µm);
Length of the flagellum I	4.1–19.0 µm	(11.4 µm);
Length of the flagellum II	6.2–15.0 µm	(10.2 µm);

Discussion

This parasite is typically spindle shaped and devoid of a posterior tail, distinguishing it from *R. wenrichi* and *R. aurangabadensis* n. sp., which have a predominantly long tail and *R. toddi* and *R. pericopti* which have a moderately long spike.

Compared with the rest of the species, the organism shows a marked difference in the body dimensions, being larger in size than most of them, as shown in Table 2. It comes nearest in size to *R. alexeieffi*, but differs in having almost equal flagella and only one cytostomal fibril. *R. phyllophagae* also has a single cytostomal fibril, but it has a pear shaped and slightly robust body with a short spike of about 1.2 µm while the present parasite is typically spindle shaped und usually devoid of a spike, besides being much larger in size.

These differences are considered sufficient to designate it as a new species and it is named *Retortamonas nagabhushani* n. sp., after Dr. R. Nagabhushanam, Head of the Department of Zoology, Marathwada University, Aurangabad.

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Table 2

Comparative Body Dimensions of the Various Species from Insects of the Genus *Retortamonas* Grassi, 1879
(All measurements in microns)

Sr. No.	Species	Length of the body	Width of the body	Length of the spike
1	*R. gryllotalpae* Grassi, 1879	7.1–14.4 (10.7)	1/3 body length	1/4 to 1/5 body length
2	*R. agilis* Mackinnon, 1911	8.0–14.0	4.5	–
	Ludwig, 1946	6.2–11.2 (7.5)	1.5–4.3 (2.5)	– –
3	*R. alexeieffi* Mackinnon, 1912	12.0–16.0	7.0–8.0	–
	Ludwig, 1946	10.0–22.5 (13.7)	4.1–8.7 (6.2)	–
4	*R. belostomae* Brug, 1922	–	–	–
5	*R. blattae* (Bishop, 1931)	6.0–9.0	5.0–7.0	–
	Bhaskar Rao, 1968	3.5–18.5 (10.8)	1.5–12.0 (5.7)	–
6	*R. phyllophagae* Travis and Becker, 1931	4.7–9.5	4.2–4.9	1.2
	Laird, 1956	4.5–11.5 (8.2)	2.6–6.3 (3.8)	–
7	*R. termitis* Kirby, 1932	3.0–6.5 (5.0)	2.5–4.5 (3.5)	–
8	*R. caudacus* Geiman, 1932	6.0–18.0	3.0–6.0	1.0–8.0 (5.0)
9	*R. wenrichi* Stabler, 1944	12.2–19.0 (16.0)	3.8–8.4 (5.9)	3.1–58.5 (23.2)
10	*R. pericopti* Laird, 1956	11.1–25.7 (16.1)	4.6–8.9 (6.5)	up to 5
11	*R. toddi* Bhaskar Rao, 1968	9.5–15.0 (12.7)	7.0–11.0 (8.2)	3.5–18.5 (9.8)
12	*R. aurangabadensis* n. sp.	15.4–25.7 (20.7)	8.2–17.0 (12.1)	4.6–60.1 (27.7)
13	*R. nagabhushani* n. sp.	9.8–18.0 (13.7)	6.2–9.2 (7.6)	–

Species: *Retortamonas nagabhushani* n. sp.
Host: *Gryllotalpa africana*
Habitat: Midgut
Locality: Aurangabad, Maharashtra, India.

The type slides are deposited in the Protozoology section, Department of Zoology, Marathwada University, Aurangabad (M. S.), India.

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RÉSUMÉ

La morphologie est décrite de deux espèces nouvelles du genre *Retortomonas*, ainsi que de *R. wenrichi* Stabler 1944, en provénance du contenu de l'intestin de *Gryllotalpa africana*. On a constaté la présence chez *R. wenrichi* d'une structure cylindrique nodeuse derrière le noyau. *R. aurangabadensis* n. sp. est caractérisée par le corps boursiforme avec une longue queue en ruban, le complexe du blépharoplaste composé de trois granules localisé latéralement par rapport au noyau, le cytostome entouré par trois fibrilles, et une structure en forme de calotte subissant une forte coloration à l'extrémité antérieure du corps. *R. nagabhushani* est caractérisée par le corps fusiforme sans queue, une paire de blépharoplastes et une seule fibrille cytostomale.

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A New Microsporidian, *Pleistophora eretesi* n. sp. from *Eretes sticticus* (L.) *(Dytiscidae, Coleoptera)*

Synopsis. A new species of a microsporidian parasite, *Pleistophora eretesi* n. sp. from the epithelial cells of the midgut of *Eretes sticticus* (L.) (*Coleoptera, Dytiscidae*) is described.

During the period 1972–1973 we frequently came across numerous spores of a microsporidian parasite belonging to the genus *Pleistophora* [1] from the epithelial cells of the midgut of *Eretes sticticus* (L.) *Coleoptera, Dytiscidae*) which is considered new to science for which the name *Pleistophora eretesi* n. sp. is proposed.

Material and Methods

The insects were collected on numerous occasions from the fresh water ponds and lakes near about the University Campus. Smears of the infected part were either air dried and fixed in methyl alcohol and stained in Giemsa's solution or wet fixed in Carnoy's fluid, hydrolysed for 10 min in 1 N HCl and stained either with Heidenhain's iron haematoxylin or according to the Feulgen's technique. Smears were also stained according to the PAS technique to reveal the presence of the polar cap.

Observations

Pleistophora eretesi n. sp.

Host: *Eretes sticticus* (L.) (*Coleoptera, Dytiscidae*)
Site of infection: Epithelial cells of the midgut
Type slide: Department of Zoology, Andhra University, Waltair, India.

About 2–3% of the insects examined revealed infection with a microsporidian parasite belonging to the genus *Pleistophora*. The infection was present in the epithelial cells of the midgut.

[1] Sprague (1971) states that *Plistophora* Labbe, 1899 is a junior synonym of *Pleistophora* Gurley, 1893 and should be discarded.

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Spores. The spores are oval in outline measuring 7.2 × 3.2 μm. There are two vacuoles, a smaller anterior one and a larger posterior one (Fig. 1). When the spores are stained according to the PAS technique a PAS positive polar cap is seen at the anterior end (Fig. 2). The polar filament could also be made out along the margin. When stained with Giemsa or Heidenhain's iron haematoxylin after an initial hydrolysis in 1 N HCl for 10 min, the sporoplasm extends in the form of a band extending in between the two vacuoles (Fig. 3). Feulgen stained spores showed two dot-like nuclei which are vesicular in nature. The polar filaments are released by the addition of a drop of hydrogen peroxide to the air dried smears. The polar filaments are uniformly thin and measure 35.0–45.0 μm in length. The pansporoblasts are irregularly rounded and

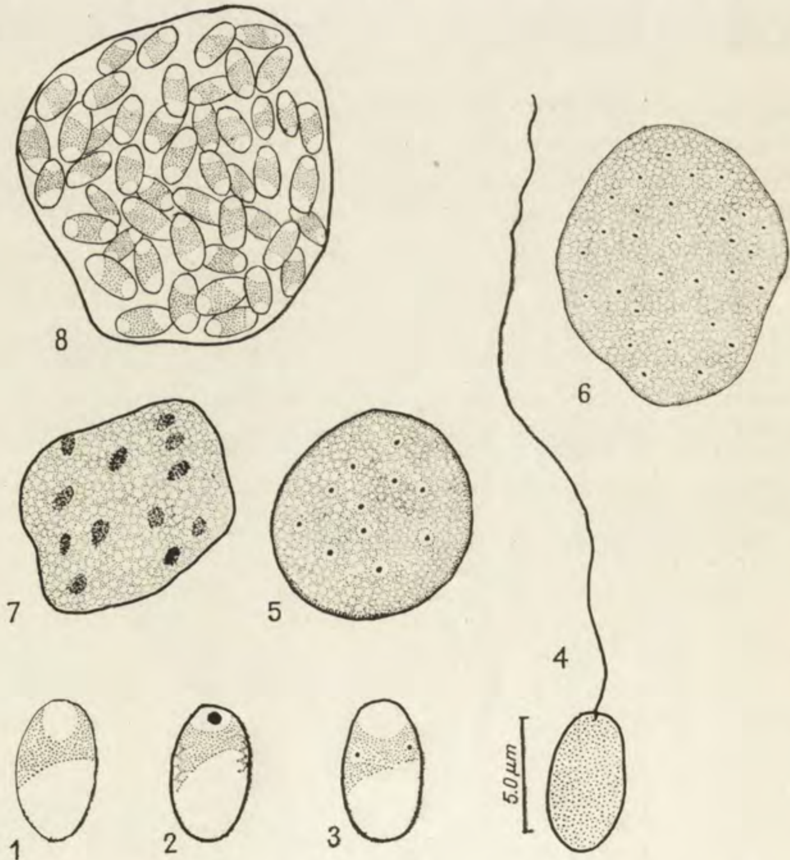

Fig. 1–8. ***Pleistophora eretesi*** n. sp. 1 — A fresh spore, 2 — A spore stained according to the PAS technique, 3 — A spore stained by the Giemsa's stain, 4 — A spore with polar filament fully released, 5 — A sporont showing 12 nuclei, 6 — A sporont showing 30 nuclei, 7 — A schizont showing 12 nuclei, 8 — A pansporoblast showing about 50 spores

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the largest observed measured 35.0 μm in diameter and contained 50 spores (Fig. 8).

Schizonts of different sizes showing variable number of nuclei were observed. The cytoplasm is coarsely alveolated and lightly stained when compared to that of the sporonts. The chromatin material of the nucleus is dispersed over a wider area and there was no endosome. The largest schizont observed measured 24.0 × 18.0 μm and contained 20 nuclei.

Sporonts of different sizes containing variable number of nuclei have been observed. They are rounded or oval and those measuring 19.0 μm in diameter contained eleven nuclei. The largest sporont observed measured 28.0–24.0 μm and contained 30 nuclei (Fig. 6). The cytoplasm is finely alveolated and a deeply stained centrally placed endosome surrounded by a clear halo is present. A clear nuclear membrane has not been observed.

Discussion

The genus *Pleistophora* Gurley includes 32 species reported from insect hosts but a perusal of the literature (among others Weiser 1961) shows that there has so far been no record of a microsporidian parasite from the present host and hence it is considered new to science for which the name *Pleistophora erestesi* n. sp. is proposed.

Diagnosis

From the epithelial cells of the midgut of *Eretes sticticus* (L.) (*Coleoptera, Dytiscidae*). Each pansporoblast gives rise to more than 16 spores. Spores oval in outline measuring 7.2 × 3.6 μm. Polar filaments uniformly thin measuring 35.0-45.0 μm in length.

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We are thankful to Prof. K. Hanumantha Rao, Head of the Department of Zoology for the excellent facilities provided to carry out this work. One of us (C. K.) is grateful to the Council of Scientific and Industrial Research for placement as a Pool Officer during the tenure of which this work has been carried out.

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RÉSUMÉ

Une nouvelle espèce de microsporidien est décrite, *Pleistophora eretesi* n. sp., parasitaire des cellules épithéliales de l'intestin du *Eretes sticticus* (L.) (*Coleoptera, Dytiscidae*).

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The Regrowth of Oral Structures in *Dileptus cygnus* after Partial Excision

Synopsis. An analysis of proboscis regrowth was performed by means of light and electron microscopy. The proboscis of *Dileptus cygnus* regrows *in situ*, while remaining mouthparts are still able to function. Observations on living cells revealed that growth takes place in the proximal portion of the growing proboscis. Formation of new kinetosomal elements for the growing proboscis occurs within an area located at the base of the proboscis. Resorption of somatic ciliature was found on the dorsal side of the proboscis and around the circumcytostomal kinety. Proboscis regrowth as an independent morphogenetic process is discussed, as well as existence of a characteristic pattern of proliferation and resorption of kinetosomes that reflects the process.

Ciliates respond in three different ways to damage to the mouthparts. Usually, a damaged mouths is replaced by a new set oral structures — a reaction identical to that after excision of whole mouth. This was reported for *Condylostoma* (Suhama 1957), *Tetrahymena* (Frankel 1960), *Stentor* (Tartar 1961), *Urostyla* (Jerka-Dziadosz 1963) and many others. Sometimes damage to the mouthparts seems not to be "noticed" by the cell. In *Hypotricha*, when a portion of the AZM is removed or destroyed by irradiation, the defective mouth may persist up to the next division (in *Urostyla* — Jerka-Dziadosz 1963) or may last for several generations (in *Euplotes* — Wise 1965). Finally, ciliates may repair *in situ* the damaged oral structure.

There are few observations concerning formation *in situ* of lost portions of the mouth. As a rule, regeneration *in situ* follows minor damage to the mouth, such as deciliation of membranellar band in *Stentor* (Tartar 1968). Cilia regrow *in situ* if the kinetosomes of membranelles are not destroyed. If deciliation does cause damage to kinetosomes, a new

[1] This investigation was supported by the Polish Academy of Sciences, research grant No. PAN 22/II, 3.

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set of oral structures is formed in primordia. Sometimes, however, Ciliates are observed to form *in situ* large portions of the mouth. Tartar 1961 reported single cases of formation of pharyngeal structure in *Stentor* when only the membranellar band was left, and formation of a membranellar band in contact with the buccal pouch only. Jenkins and Sawyer (1970) observed *in situ* formation of oral membranelles in *Blepharisma*. Doublets of *Paramecium* are also able to repair *in situ* drastic damage of one of oral sets (Hanson 1955).

The process of regeneration *in situ* of oral parts has never been studied in detail. The aim of presented study is analysis of this morphogenetic process. Regrowth of the proboscis in *Dileptus* represents a very convenient model for studying the *in situ* regeneration.

The analysis of proboscis regrowth is based on observations made on living cells, as well as on cell preparations for light and electron microscopy.

Material and Methods

The present study on regrowth of the proboscis was performed on the ciliate *Dileptus cygnus* Clap. et Lachm., 1859. The ciliates were kept on Petri dishes in Pringsheim solution. A filtered and concentrated mass culture of *Colpidium* was supplied as food. The animals were fed one day before they were used for experiments.

Three types of operations were made: (1) The proboscises were cut off at different distance from the cytostome. Proboscis regrowth rate was observed. (2) The proboscises were cut off at different distances from the cytostome, and a large posterior part of the cell posessing the whole nuclear apparatus was also cut off. The rate of proboscis regrowth was observed in anuclear fragments. (3) The proboscises were cut off at different distance from the cytostome and the ventral band of remaining stump was also incised. Changes in the distance from the incision to the top or to the base of the regrowing proboscis were measured.

The operations were made by hand, using a microscalpel, on the cells in culture dishes. Then single operated cells were isolated into depression slides, with an amount of water not less than 4–5 drops (0.5 ml). Depression slides were put under the Leitz microscope and animals were photographed every 30 min. Each time 3–4 pictures were taken. Usually animals were photographed 0, 30, 60, 90 and 120 min after the operation. In some cases observations were prolonged up to 24 h and even 48 h. The rate of proboscis regrowth was measured using the negatives of photographs taken during the regeneration. The negatives were projected in constant magnification on paper sheets and cell contours were outlined with pencil. The length of different parts of the ciliate body was then measured on the outlines.

The preparations of the ciliates with a regrowing proboscis were stained with protargol after Tuffrau (1964), or with osmium after Grain and Golińska (1969). For these, the operations were performed in a mass culture during a 30 min

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interval. Each sample contained 50–100 individuals. The samples were fixed in subsequent periods: 0–30, 30–60, 60–90 and 90–120 min after the operation. The Leitz Ortholux microscope was used to observe and photograph the preparations.

The operated ciliates were fixed for EM observations 15–25, 25–40, 40–60, 60–75 and 75–90 min after the operation. A mixture of 2% OsO_4 and 6% glutaraldehyde dissolved in cacodylate buffer was used for fixation. During fixation preparations were kept on ice for one hour. Then they were washed and grouped on 0.5% agar, then embedded in Epon according to conventional methods. The material was sectioned on an LKB ultramicrotome and contrasted with uranyl acetate and lead citrate. Sections were examined under transmission electron microscope JEM 100 B.

Results

Before giving an account of the process of repair of oral parts we wish to describe in detail the structure of mouthparts in *Dileptus cygnus*. This description, when compared to the previous one (Grain and Golińska 1969), contains some supplementary data.

In *Dileptus* the cytostome is a deep, permanent depression, situated roughly in the middle of a nonciliated cytostomal field (Fig. 1). The cytostome is encircled by a double palisade of nemadesms. The internal basket of nemadesms is joined to the surface in the territory of the cytostomal field. The external basket joins the kinetosomes of the oral

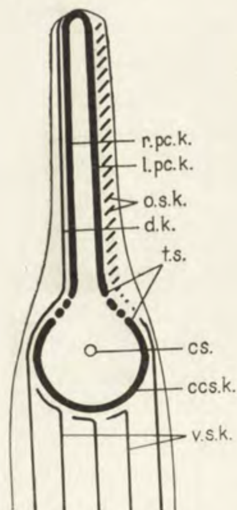

Fig. 1. Arrangement of oral and somatic kineties on the ventral side of the ciliate *Dileptus cygnus*. **Heavy lines designate oral kineties, slender lines designate somatic kineties.** Explanations: right paracytostomal kinety (r.pc.k.), left paracytostomal kinety (l.pc.k.), oblique somatic kineties (o.s.k.), dense somatic kinety (d.k.), transitional segment of oral kinety (t.s.) cytostome (cs), circumcytostomal kinety (c.cs.k.), ventral somatic kineties (v.s.k.)

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kinety on the borderline between the cytostomal field and the somatic region on the ventral side of the *Dileptus* body. On its ventral side the cytostomal field is elongated into the so-called ventral band, that occupies the entire ventral side of proboscis.

The infraciliature of *Dileptus* may be divided into oral and somatic, a distinction that is based on the structure of associated fibers and the position of kineties with regard to cytostome. The somatic infraciliature is composed of kinetosomes all of which are ciliated, and which are endowed with identical complements of fibers, with each containing a transverse, postciliary and rootlet fiber, and a kinetodesma (Fig. 2). Somatic kineties are arranged meridionally, with the exception of short oblique kineties on the left side of the proboscis (Fig. 1). On the right side of the body and proboscis there is a kinety designated by us a dense somatic kinety (Fig. 1). Kinetosomes are very densely packed in the segment of the dense kinety that runs in the area of the proboscis up to its apex. The remaining portion of the dense kinety does not differ from its neighbours.

The oral infraciliature is located exclusively along the line bordering the cytostomal field (Fig. 1). The basic unit of oral infraciliature is represented by a kinetosome lacking its cilium, and oriented in such a way that its transverse fiber is directed towards the cytostome (Fig. 2). The whole cytostomal field is provided with these transverse fibers and some of them enter the cytostome itself. Another common feature of the oral infraciliature is a lack of kinetodesma.

Although the oral infraciliature forms a continuous line around the cytostomal field, and probably represents a single oral kinety, we refer to it as to the three oral kineties, because these three segments of oral kinety have a different structure. These oral kineties were described for *Dileptus visscheri* (Kink 1976) as the circumcytostomal kinety and the right and left paracytostomal kineties.

The circumcytostomal kinety is situated all around the cytostomal field, except for its elongated portion on the proboscis (ventral band). This kinety is a single row of nonciliated kinetosomes, each bearing nemadesma and a transverse fiber (Fig. 2, Pl. I 1). Somatic kinetosomes of curved ends of ventral somatic kineties may be found in close proximity to the oral kinetosomes (Pl. I 1, 2). Sometimes connnections of dense material were observed between proximal portions of oral and somatic kinetosomes (Grain and Golińska 1969). Such pairs are not found regularly along the circumcytostomal kinety, as most of the oral kinetosomes have no close somatic neighbour.

Towards the dorsal side the circumcytostomal kinety on the right side becomes the right paracytostomal kinety, and on the left it becomes the

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left paracytostomal kinety. Paracytostomal kineties constitute the borders of the ventral band, and each is made up of a single row of kinetosomal pairs (Fig. 2, Pl. II 5, 6, Pl. III 9, 10). These pairs contain one nonciliated kinetosome close to the ventral band and bearing the transverse fiber,

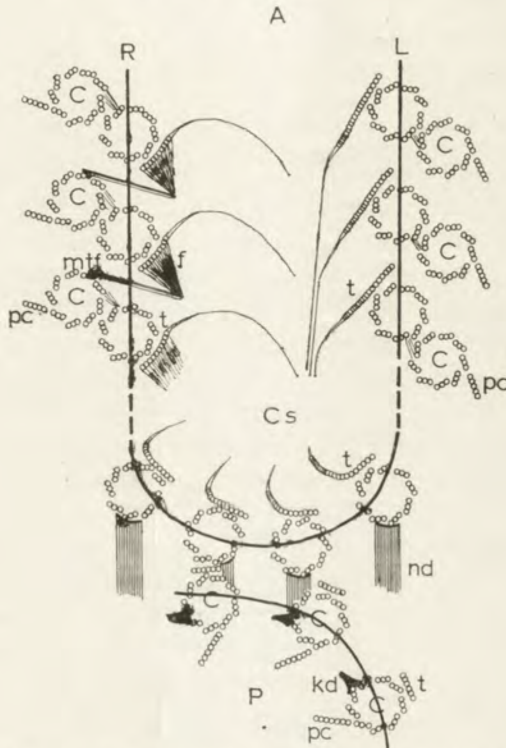

Fig. 2. Structure of oral and somatic kineties of *Dileptus cygnus*. Heavy lines present the disposition of kineties. The U-shaped kinety is the oral kinety, below it there is a curved segment of a ventral somatic kinety. anterior (A), posterior (P), right (R), and left side of the cell (L). Cytostome (Cs), cilium (C), transverse fiber (t), postciliary fiber (pc), kinetodesma (kd). The additional fiber that joins pairs of the right paracytostomal kinety is marked (mtf) for its portion composed of microtubules and filaments, and for its striated filamentous part (f)

and one ciliated kinetosome bearing the postciliary fiber. There are always complicated interconnections between the proximal ends of kinetosomes of each pair. Transverse fibers that are derivatives of left paracytostomal kinety are exceptionally long and form the so-called central fiber in the territory of ventral band (Fig. 2, Pl. II 6, Pl. III 8). Transverse fibers belonging to the right paracytostomal kinety run between the toxic trichocysts and end on central fiber (Pl. II 5, Pl. III, 8). Moreover, the kinetosomal pairs of the right paracytostomal kinety are linked together with an additional fiber (Pl. II 5, 7, Pl. III 9, Fig. 2). This fiber has a filamentous striated portion that is connected with the base of the

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transverse fiber of one kinetosomal pair, and the complex filament-microtubular portion that joins the ciliated kinetosome to the next kinetosomal pair.

Somatic kinetosomes, as well as oral kinetosomes, possess transverse and postciliary fibers. These fibers differ, however, in number of microtubules. The number is constant for all somatic kinetosomes. The postciliary fiber contains seven tubules arranged in one row, while the transverse fiber has one row of six long tubules and one row of very short three tubules (Fig. 2, Pl. III, 11). Oral kinetosomes have a postciliary fiber only when they belong to a paracytostomal kinety. This fiber has only five tubules (Fig. 2, Pl. III, 9, 10). Transverse fibers joined to oral kinetosomes have microtubules arranged in a single row, the number of microtubules varying with position on the circumference of cytostomal field. Kinetosomes of the left paracytostomal kinety have a transverse fiber consisting of 20–22 microtubules, those of right paracytostomal kinety of 10–11 microtubules (Pl. III, 8). Kinetosomes belonging to the circumcytostomal kinety have a number of microtubules varying from 12 to 18. It seems probable, that within the circumcytostomal kinety there is a gradient in the number of microtubules in the transverse fiber from 18 on the left side to 12 on the right side.

This review of the structure of the oral ciliature in *Dileptus* makes clear, that excision of proboscis means excision of a highly specialized portion of the mouth represented by the ventral band and its infraciliature.

Observations on Living Cells

The length of the proboscis in trophic cells of *Dileptus* cygnus varies in a wide range. The longest proboscis that has been measured was 600 μm long, this being not a maximum. As a rule, the better the culture conditions are, the longer the proboscis of *Dileptus* is. Only cells with long proboscises were chosen for the operations.

Preliminary observations revealed that partial or complete excision of the proboscis does not stop the functioning of pharyngeal mouthparts. *Colpidia* immobilized or killed by high temperature served as a food. *Dileptus* cells after excision of the proboscis were able to swallow these colpidia. Swallowing was observed immediately after the operation, as well as during the whole process of proboscis regrowth.

Experimental analysis of the regrowth of the proboscis consist of measurements of regrowing proboscises in normal and anucleate cells, and of measurements of regrowing proboscises with an additional incision made on the ventral band.

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The first series of measurements was performed on 15 cells. The proboscis was cut off at different levels. Sometimes the whole proboscis was excised, while sometimes a stump up to 150 μm long remained. Long lasting observations were performed on six of these cells. These observations showed, that regrowth of the proboscis takes a very long time. The process of regrowth of the proboscis ended sometime between 6.5 and 24 after the operation in these cells. The rate of regrowth varied during its course. The highest rate was observed during the first hour after the operation, with a maximum at 90 μm/h. Later on the rate of regrowth distinctly decreased (Fig. 3). Measurements were also performed every 30 min during the first 90 min after operation on another group of nine cells. Results are presented on Fig. 4 I. Comparison of these data shows that cells with the proboscis cut off at the same level may regrow their proboscises at different rates. On the other hand, proboscises excised at different levels may regrow at the same rate (see also Fig. 3). There is no distinct relation between rate of regrowth and the length of the remaining stump of proboscis.

The second short series of experiments deals with the ability of anucleate fragments to regrow their proboscis. Anucleate anterior fragments almost as large as halves of the cell were used. To obtain such large anucleate fragments bisection was performed on animals while their nuclear apparatus was incidently displaced posteriorward, or on dividing animals with a condensed macronucleus. The proboscis of the anucleate fragment was cut off immediately after bisection. The results of measu-

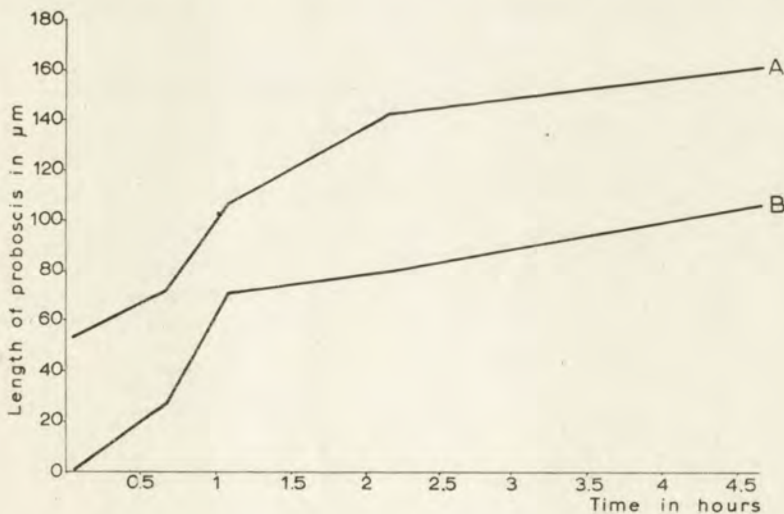

Fig. 3. The rate of regrowth of the proboscis of *Dileptus cygnus* after partial (A) and total (B) excision. Each curve represents a single cell

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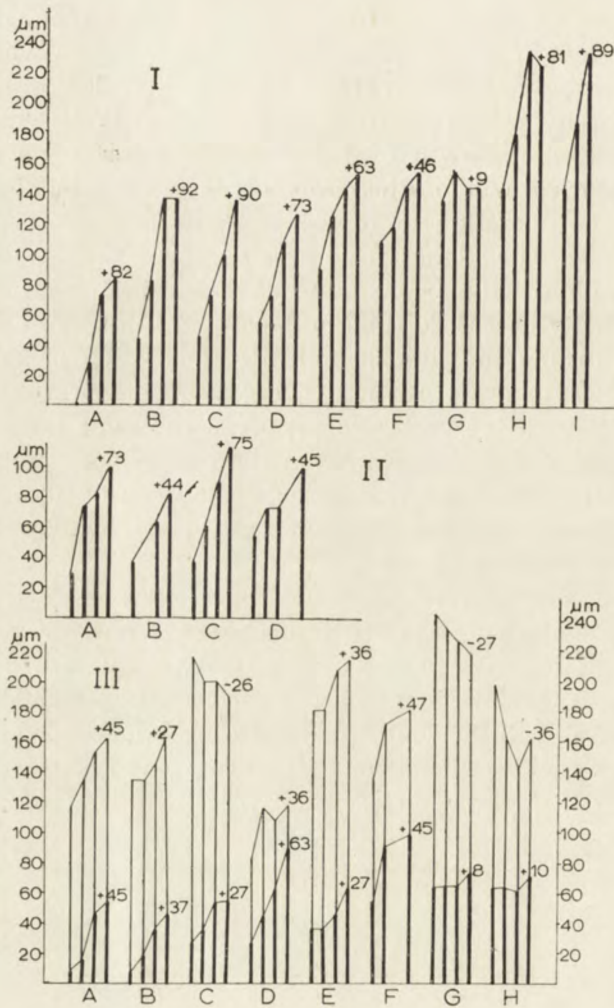

Fig. 4. Diagram I demonstrates the rate of regrowth of proboscis of *Dileptus cygnus* after excision. The vertical axis represents the length of the proboscis in µm. Each group of measurements (A, B, C ....) represents an experiment on a single cell. The first measurement was performed immediately after the operation. The distances between subsequent heavy lines in each group correspond to 30 min period. The number on top of the heavy lines indicate the total lengthening of the proboscis within a 90 min period. Diagram II demonstrates the rate of regrowth of the proboscis in ciliates without a nuclear apparatus. Designations are like those in Diagram I. Diagram III demonstrates the rate of regrowth of incised proboscis stumps. Heavy vertical lines in each case (A, B, C...) correspond to the segment from the base of the proboscis to the incision. Slender lines represent the segment from the incision to the top of the stump. The numbers at the top of the heavy lines in each case correspond to the lengthening of the segment from the base of proboscis to incision. The numbers at the top of last slender line represent in each case lengthening (+) or shortening (—) of the whole stump

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rements on four such fragments are presented on Fig. 4 II. It is clear that proboscis regrowth may proceed at a normal rate in anucleate cells, at least during first 90 min after the operation.

The third series of measurements was made to resolve the problem of whether growth of the proboscis is uniform over its entire length. If it is not, the growth may be localized at the base or at the apex of growing proboscis. In the experiment a portion of the proboscis was cut off, and then an incision was made on the remaining stump. The incision was made in such a way that the ventral band was sectioned. Then, if the distal portion twisted before the wound healed, the incision was visible up to three hours after the operation (Pl. III 13–16). If there was no twisting, the incision became invisible in less than 30 min. This incision served as a marker to measure the length of proximal and distal segments of the growing proboscis. The measurements performed on eight cells in which the incision was clearly visible for 90 min are presented on Fig. 4 III. The two segments of the proboscis, the proximal segment from the cytostome to the incision and the distal segment from the incision to the apex behave differently during proboscis regrowth.

The distal portion of proboscis may behave in four different ways. It may remain of the same length (Fig. 4 III, cases A, F), it may elongate a little (case E), it may elongate at first and then shorten (case D), or it may shorten (cases C, G, H, see also Pl. IV). Observations performed longer than 90 min revealed that elongation of the distal segment is restricted to the period immediately following operation.

It is clearly visible in the Fig. 4, III that if growth of the proboscis as a whole occurs, it is due to the growth taking place in its proximal segment (cases A, B, D, E, F). If there is shortening of the proboscis as a whole, it is due to the shortening of its distal portion (cases C, G, H), and takes place in spite of growth that simultanously occurs in its proximal part.

The proximal portion grow in the same manner as the whole stump in the previous experiments. The rate of growth was, however, much slower than in series I and II. It is unknown whether the low speed of growth was caused by the presence of the incision, or by the fact that the whole stump was much longer than that in previous series. We believe, however, that the presence of an incision have some inhibitory effect on elongation of distal portion of proboscis. The regrowth in previous series could be especially quick during first hour after operation due to cumulation of this elongation of the distal portion and regrowth of the proximal portion of proboscis. Moreover, the presence of an incision seems to cause the shortening of the distal portion of the stump.

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Images of the Growing Proboscis Obtained in EM and Light Microscopy

The process of proboscis growth consists of elongation of its distal portion and of regrowth of its proximal portion. The elongation of the distal portion of proboscis proceeds without the formation of new kinetosomal elements, while the regrowth in proximal portion is accompanied by formation of new kinetosomes. The following paragraphs will present the differences which could be noticed in the growing proboscis when compared to a normal one, beginning with the apex of the proboscis and ending at the circumcytostomal kinety. This does not reflect, however, the temporal sequence of events, because all of these events may procede simultanously in the same cell.

Formation of the Apical Portion of a Growing Proboscis

In trophic cells of *Dileptus* the proboscis is thick at its base, and thins gradually towards the apex. Sections of proximal and distal portions of the proboscis show that the differences of circumference are mostly situated in the somatic portion of proboscis while the ventral band has a nearly constant width (Pl. V). In the proximal portion of proboscis there are 6–8 somatic kineties, but only the paracytostomal kineties, dense kinety and oblique rows reach the apex. The remaindes of the somatic kineties end at different levels on the narrowing proboscis. The narrow part of the proboscis, like that presented on Pl. V 17, is comparatively long in trophic cells and constitutes 1/5–1/3 of the length of proboscis. In this narrow part distances between kinetosomes in paracytostomal kineties and the dense kinety and distances between oblique rows increase gradually towards the apex of proboscis.

After partial excision of the proboscis a new apex situated at the end of the stump has too many kineties with kinetosomes too crowded in them when compared to the normal apex. The elongation of the whole distal portion of the stump and reduction of its somatic part is needed for restoration of a normal apex.

Observations concerning an increase of the distance between kinetosomes on the distal portion of the growing proboscis stump were performed on protargol impregnated preparations. In preparations made 0–30 min after the operation, only eight cells out of 30 were found to have a uniform density of kinetosomes all along the stump. The remaining cells showed a gradual increase of space between kinetosomes in the distal portion of the growing proboscis. In preparations made 30–60 min after the operation cells with a uniform density of kineto-

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somes on the stump were found occasionally only. It seems that elongation of the distal portion of the growing stump takes place during first 30 min after operation.

Observations concerning the reduction of the somatic portion of the new distal part of proboscis were made on protargol preparations and on EM preparations. Protargol preparations revealed that when a new distal part is elongated only 3–4 somatic kineties reach the apex. The rest of the somatic kineties cannot be traced up to the new apex. In those "additional" kineties which reach the apex, distances between kinetosomes become greater towards the apex (Pl. VI 23).

Electron microscopy shows images of resorption of cilia in distal parts of the growing proboscis (Pl. V 19). There are cilia beneath the surface of proboscis and attached kinetosomes are sometimes visible. Those cilia are situated mostly in the somatic portion of proboscis. Because of the position of resorbed cilia and the lack of resorbed nonciliated kinetosomes those electronmicrographs seem to be images of resorption of somatic ciliature. Images of resorption of cilia in distal portion of the proboscis were found on all preparations, also on those made very early, 15–25 min after operation.

Images of growing proboscises obtained in the EM showed no proliferation of oral kinetosomes within paracytostomal kineties. No formation of new oral kinetosomes was found even in the apical portion, where there are greater spaces between kinetosomal pairs. In the proximal part of the growing proboscis the proliferation of somatic kinetosomes was occasionally visible. This was found in all somatic kineties including the dense kinety and oblique rows. In the dense kinety an intense proliferation of somatic kinetosomes was found at the base of the proboscis.

Changes of Structure at the Circumference of Cytostomal Field

While the proboscis grows the remaining mouthparts show a change in their structure compared to the mouth of the trophic cells. The curved segments of ventral somatic kineties that normally associate with the circumcytostomal kinety disappears (Pl. I 1, 2). Images of cilia resorption were found (Pl. VI 20, 22). Also, an intense proliferation of oral kinetosomes was found all around the circumcytostomal kinety (Pl. VII, 25 a, b). All the other features of the oral parts seemed unchanged — cytostome, internal basket of nemadesms, external basket of nemadesms, its kinetosomes and transverse fibers directed towards the cytostome — all this did not look different than in trophic cells.

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The disappearance of curved segments of ventral somatic kineties was first noticed in cells stained with osmium. This method makes visible only kinetosomes equipped with cilia. The images obtained of oral parts are identical with those stained with hematoxylin (Golińska 1974). On trophic cells stained with osmium the ventral side of the cytostomal field seems to be bordered with segments of somatic kineties only (Pl. I 2). The circumcytostomal kinety is not visualized by this method. Cells with their proboscises cut off stained 30–60 min after operation have no curved ends of somatic kineties bordering the cytostomal field; ventral somatic kineties end abruptly at the imaginary line limiting the cytostomal field (Pl. VI 21). This change is not visible in preparations impregnated with protargol (Pl. VI 23). The protargol method visualizes nonciliated kinetosomes as well as these equipped with a cilium. In normal and operated cells the borderline on the ventral side of cytostomal field usually appears as a coarse dark line, and single kinetosomes may scarcely be distinguished. In normal cells the curved ends of ventral somatic kineties are hard to distinguish from the circumcytostomal kinety, so their disappearance after proboscis excision cannot be noticed.

EM observations confirm a lack of cilia at the ventral margin of cytostomal field. The section just above the circumcytostomal kinety shows no cilia in the depression between the oral field and the ventral somatic part of the body (Pl. VI 20). Images of cilia resorption were found near the circumcytostomal kinety. These resorbed cilia drawn under the surface of the cell are especially numerous in preparations made 40–60 min after operation and may be found in every preparation made from 15–25 min to 2 h. It is impossible to decide whether there exists some distinct period of cilia resorption because we do not know the rate at which cilia desintegrate in the cytoplasm. We suppose, that the resorption involves somatic cilia of the curved segments of ventral kineties.

Oral kinetosome proliferation was observed on the outer margin of circumcytostomal kinety (Pl. VII 25 a, b), and only there. Images of kinetosome proliferation were found in the very place where somatic kinetosomes of curved segments had been, and were found in close proximity to images of somatic cilia resorption. Very intense proliferation was found in cells fixed 15–25 and 40–60 min after the operation. Every section through several kinetosomes of the circumcytostomal kinety showed some new oral kinetosomes. About 2 h after the operation proliferation of oral kinetosomes was not so intense and new kinetosomes were scarcely found along the outer margin of cytostomal field.

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Folding of Kineties at the Base of Proboscis

Folded oral kineties are often seen near the base of the stump in cells stained with protargol during the first hour after the proboscis was partially excised (Pl. VII 26, 28). This folding is always restricted to the area where paracytostomal kineties join the circumcytostomal kinety. The segment of dense somatic kinety that lay within this area may be folded too. When cells are fixed later than during first hour after operation, their kineties are no longer folded at the base of proboscis.

EM observations show that folding covers the basal portion of paracytostomal kineties and the transitional segments lying between the paracytostomal and circumcytostomal kinety. In normal cells the transitional segments are built of kinetosomal pairs (Pl. I 3). Close to nonciliated kinetosomes bearing nemadesma there are situated ciliated kinetosomes that possess the postciliary fiber only. Within the transitional segment connections between the ciliated and nonciliated kinetosome in every pair are not so distinct as in paracytostomals. Moreover, not every nonciliated kinetosome has its ciliated neighbour. In osmium stained preparations transitional segments look iike curved segments of the paracytostomal kinety (Pl. I 4).

Dileptus cells with their proboscises cut off show no change in fine structure of infraciliature within the folded area when compared to normal cells, Except for folding, transitional segments and basel portions of the paracytostomal kineties look perfectly normal (Pl. VII 27). No pictures of kinetosomal proliferation were found, except for proliferation within the dense kinety. Intense proliferation of oral kinetosomes takes place just below area of folding. This is true for proliferation of oral kinetosomes along the circumcytostomal kinety and also for somatic kinetosomes within the dense somatic kinety. Protargol staining shows that during proboscis regrowth this kinety is closely packed with kinetosomes in all regions anterior to the middle of the *Dileptus* body (Pl. VI 24).

Discussion

The data obtained show that proboscis regrowth is a distinct morphogenetic process. This kind of regeneration of oral and somatic structures is different than stomatogenesis and much more complicated then we had expected the process of regrowth *in situ* to be. We intend to discuss the results as follows: first the course of proboscis regrowth, then the pattern of kinetosomal proliferation and resorption characterizing the

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lengthening of the proboscis, and finally the comparison of this process with stomatogenesis.

The data obtained on proboscis lengthening in *Dileptus* permit us to reconstruct the manner in which the regrowth occurs. Lengthening of the whole proboscis is due to the elongation of its apical portion together with growth at its base. The elongation of the apical portion of the remaining stump is probably restricted to a short period following the operation, and is not accompanied by formation of new kinetosomes. The elongation of apical portion was observed on light microscope preparations where distances between kinetosomes became greater towards the apex. Existence of apical elongation may also be deduced from comparison of unusually high rate of regrowth during first hour after operation with the much lower rate of regrowth later on, and also with the regrowth of the incised stumps. It is not clear whether there is growth of the surface, or whether the elongation is simply a result of proboscis narrowing.

The growth at the base of proboscis lasts several hours and involves formation of new kinetosomes. The existence of localized growth at the base of the proboscis stump was deduced from several facts. It was observed that growth of a stump with an incision is due to growth of its proximal portion, from the cytostome to the incision. In addition, the formation of new kinetosomes for the growing proboscis was found to occur always within the area located below the ventral band, regardless of the length of the proboscis stump. Moreover, the folding of oral kineties at the base of ventral band observed during the first hour after operation may disappear afterwards, indicating an initial basal crowding of kinetosomes with subsequent distribution over the growing proboscis.

We suppose that oral kinetosome formation is preceded by the resorption of the curved ends of the ventral somatic kineties. The resorption of somatic kineties close to the site of oral kinetosome formation was also observed during stomatogenesis in *Dileptus* (Golińska and Grain 1969, Golińska 1972). During stomatogenesis resorption of somatic cilia serves probably to prepare future, nonciliated cytostomal field. We suppose, that somatic kinetosomes were resorbed all around the circumcytostomal kinety during proboscis regrowth to prepare a site for oral kinetosomes to proliferate.

It seems probable that new oral kinetosomes formed all around the circumcytostomal kinety may move towards the ventral band before their differentiation into elements of the oral kinety. We do not suppose them to be inserted between elements of circumcytostomal kinety, because no enlargement of the external basket of nemadesms was observed. New kinetosomes are probably neither differentiated nor incorporated into an

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oral kinety before they reach the transitional segment. Folding of basal portions of paracytostomal kineties together with the transitional segments seems to be due to a great number of new kinetosomal elements thrust at the base of the transitional segments.

The folding observed during the first hour after the operation indicates that growth, although rapid during this period, is still slower than the formation of new oral elements. Subsequent smoothing of the folds may be a sign of attainment of a balance between rate of formation of new kinetosomes and rate of growth of the surface at the base of the growing proboscis. Inclusion of new elements at the base of the growing proboscis seems very profitable for animals. Immature elements of the mouth that probably are not yet functioning are situated in between the paracytostomal and circumcytostomal kinety, leaving the growing proboscis as well as pharyngeal parts ready to function.

A similar mechanism of proboscis lengthening was found to follow stomatogenesis. Kink (1976) reported that growing excysted cells of *Dileptus visscheri* have an area of growth localized at the base of proboscis. An intense proliferation of oral and somatic kinetosomes in the area below the base of ventral band was documented during this growth of mature oral parts. Kink has concluded that this localized area of proliferation creates new kinetosomes for proboscis lengthening, cytopharyngeal basket enlargement, and elongation of the whole body. During proboscis regrowth the area of proliferation is localized in the same place where excysted cells have their area of proliferation. However, in the case of proboscis regrowth this area supports kinetosomes for proboscis lengthening only. We have some unpublished data indicating that during growth following stomatogenesis in the processes of division and regeneration there is an identical area of kinetosomal proliferation situated all around the circumcytostomal kinety.

The process of proboscis growth that normally follows stomatogenesis was separated from stomatogenesis itself with the aid of inhibitors. Puromycin (Golińska 1974) was found to inhibit proboscis growth during regeneration without inhibition of formation of oral parts, resulting in individuals having apically located pharyngeal parts. Actinomycin D (Golińska et Bohatier 1974, Golińska and Bohatier 1975) was found to suppress proboscis growth, without delaying regenerational stomatogenesis. Thus, in spite of the fact that growth of proboscis usually follows stomatogenesis, this growth shows much greater sensitivity to the action of the drugs than even early stages of stomatogenesis. We intend to test the effect of the drugs on the process of proboscis regrowth.

In the absence of nuclear apparatus *Dileptus* may form its oral parts

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(G o l i ń s k a 1966), although the development is limited to the first two hours after the operation, and only short proboscis may be formed. We found now, that regrowth of proboscis is possible in the absence of nucleus, at last during first 90 min after the operation.

We suppose that the proboscis grows not only in response to excision or stomatogenesis but also in normal trophic cells. Proboscis lengthening in mass cultures may be easily observed, i.e., after the addition of fresh medium. Images of oral kinetosome proliferation next to the circum-cytostomal kinety have often been observed in trophic cells. We suppose that the process of proboscis lengthening may start at any moment in the lifetime of trophic cells as a response to some change of medium. The existence of a mechanism that allows elongation of proboscis at any stage of the cell cycle indicates that a mechanism of proboscis shortening must also exist to keep the length of the proboscis in balance with the rest of the cell. That is the subject of research we are doing now.

We failed to distinguish any distinct stages of proboscis regrowth. All of the changes in the structure of a cell with its proboscis regrowing may be found simultaneously. Instead of successive stages, we have found that simultaneous proliferation and resorption of infraciliature forms a very distinct pattern on the surface of the cell during proboscis growth. The density of resorbed or formed elements may change with the intensity of growth, but there remains the same pattern for a given morphogenetical process. During proboscis regrowth there is resorption, restricted to the somatic ciliature and located on the dorsal side of proboscis and around the ventral part of cytostomal field (Fig. 5), and there is proliferation of oral as well as somatic kinetosomes situated around the ventral part of cytostomal field.

Our previous observations on stomatogenesis in *Dileptus* (G o l i ń s k a and G r a i n 1969) indicate, that in stomatogenesis the proliferation — resorption pattern is different from the pattern found for proboscis regrowth (Fig. 5). In early stages of stomatogenesis oral kinetosome proliferation begins at the dorsal side of a fragment, or dorsal side of dividing cell (G o l i ń s k a 1972), and then spreads forming left and right rows that eventually join each other on the ventral side. Resorption affects the somatic ciliature only, and is restricted to the surface between the spreading rows of oral ciliature, on the area of the future cytostomal field. In dividing cells resorption was found to occur anterior to the developing oral ciliature on the right and left side of animal.

A pattern of simultaneous formation and breakdown of the microtubular structures was reported by T u c k e r (1971) for dividing cells of *Nassula*. Our observations on *Dileptus* supply the additional information that this pattern is different for different morphogenetic processes.

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We are convinced, that any morphogenetic process that involves some change of shape in *Dileptus* is reflected in its proliferation — resorption pattern.

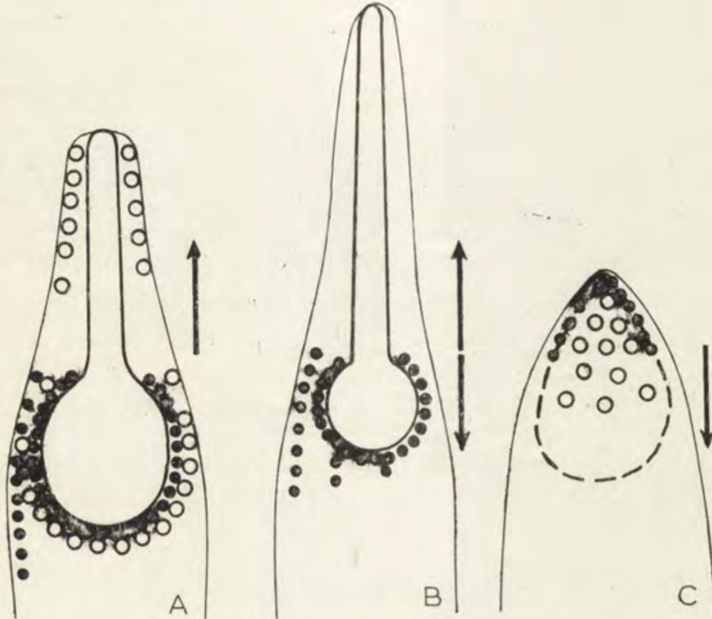

Fig. 5. Illustration of the pattern of kinetosomal proliferation and kinetosomal resorption during three different developmental processes of *Dileptus cygnus*. Proliferation of kinetosomes is marked with black dots, resorption of kinetosomes is marked with open circles. The heavy line designate the oral kinety. Arrow indicate the directions of growth of mouthparts. Case A — proliferation-resorption pattern during regrowth of the proboscis. Case B — proliferation pattern during growth of the oral apparatus in an excysted ciliate. Case C — proliferation-resorption pattern during early stages of stomatogenesis. The discontinuous line represents the course of the future oral kinety

Summary

An analysis of proboscis regrowth was performed by means of light and electron microscopy. The proboscis of *Dileptus cygnus* regrows *in situ*, while remaining mouthparts are still able to function.

Observations on living cells revealed that the rate of growth of the proboscis may be the same after total and partial excision of the proboscis. Moreover, observations on regrowth of incised stumps indicate that regrowth may be restricted to the proximal portion of the mouthparts.

Oral ciliature has never been found to proliferate on the growing proboscis. Instead, proliferation of oral kinetosomes was found all around the circumcytostomal kinety. Somatic kinetosomes were observed to pro-

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liferate mostly within an area close to the circumcytostomal kinety. Images of resorption of somatic ciliature were found close to the circumcytostomal kinety, and on the dorsal side of the growing proboscis.

It was concluded that new kinetosomes joined the kineties of the growing proboscis within an area located at the base of the proboscis. This area corresponds to the region of the growth of the proboscis.

The process of proboscis regrowth is discussed as an independent morphogenetic process which is not a stomatogenesis but nonetheless involves formation of oral kinetosomes. The proliferation and resorption of kinetosomes during proboscis regrowth form a characteristic pattern on the cell surface. The supposition is presented that each morphogenetic process which is accompanied by a change of cell shape of *Dileptus* is reflected in a characteristic pattern of resorption and proliferation of kinetosomes.

ACKNOWLEDGEMENTS

The authors thank to Dr. Joseph Frankel and Dr. Maria Jerka-Dziadosz for their help in preparing this paper, and they also thank to Mrs. Lidia Wiernicka for expert technical assistance.

RÉSUMÉ

La recroissance du proboscis de *Dileptus cygnus* a été analysée en microscopie optique et électronique. Le proboscis repousse sur place quand d'autres parties orales restent en état de fonctionnement.

L'observation des cellules vivantes révèle qua la croissance s'effectue dans la partie proximale du proboscis en reconstitution. Les éléments cinétosomiens nouveaux sont formés, pour le proboscis en croissance, dans la région située à sa base. On constate la resorption de la ciliature somatique sur le côté dorsal du proboscis, et autour de la cinétie circumcytostomienne.

La discussion concerne la recroissance du proboscis en tant que phénomène morphogénétique indépendant, ainsi que la question de la présence d'un pattern caractéristique de la prolifération et de la resorption des cinétosomes, qui reflète ce processus.

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EXPLANATION OF PLATES I–VII

Infraciliature in trophic cells of *Dileptus cygnus*

1: A section through the circumcytostomal kinety (ccs.k) and the curved segments of somatic kineties (s.k.). Oral and somatic kinetosomes are in close proximity to each other. Oral kinetosomes possess a transverse fiber (t) only. Somatic kinetosomes possess transverse fiber (t), postciliary fiber (pc) and kinetodesma (kd). Cytostomal field (cs.f) × 15 500
2: Arrangement of the ciliated kinetosomes in proximity to the cytostomal field (cs.f.). Arrows indicate the curved segments of somatic kineties. Preparation stained with osmium. × 100 obj.
3: The transitional segment of the left paracytostomal kinety (l.pc.k.) at the base of proboscis. Cytostomal field (cs.f.), central fiber (c.f.). Somatic kinetosomes (s.ks.) of the short, oblique kineties. × 12 500
4: The left paracytostomal kinety (indicated by arrows) on the base of the proboscis. The sector between two continuous lines corresponds to the fragment showed on Fig. ?. Preparation stained with osmium. × 100 obj.
5: A section through the right paracytostomal kinety (r.pc.k.) and the dense kinety (d.k.). Microfibrillar band (mf). On the left side toxicysts are visible. × 15 500
6: A section through the left paracytostomal kinety (l.pc.k.). The discontinuous line indicates the ciliated kinetosomes. Somatic kinetosomes of short oblique kineties (s.ks.). Central fiber (c.f.). × 10 500
7: Oral kinetosomes of the right paracytostomal kinety. Microtubular (mt.f.) and filamentous (f) elements build the additional fiber. × 19 500
8: A transverse section through the central fiber. Right transverse fibers (r.t.) join the right paracytostomal kinety. Left transverse fibers (l.t.) join the left paracytostomal kinety. × 25 500
9: A portion of the right paracytostomal kinety. Ciliated kinetosomes (c.ks.) and nonciliated kinetosomes (n.ks.) form pairs of kinetosomes. Transverse fiber (r.t.), postciliary fiber (pc.). A filamentous element (f) and a microtubular-filamentous element (mt.f.) together constitute the additional fiber. ×18 000
10: A portion of the left paracytostomal kinety. Ciliated kinetosomes (c.ks.) and nonciliated kinetosomes (n.ks.) are joined as the pairs of kinetosomes. Transverse fiber (l.t.), postciliary fiber (pc.), microfibrils (mf.). × 18 500
11: A section through a somatic kinety. Transverse fiber (t), postciliary fiber (pc.), kinetodesma (kd.). × 15 500

The course of growth of an incised stump.

12: The trophic, normal ciliate. Cytostome (cs.). × 10 obj.
13: The same ciliate with proboscis partially excised and with an incision (arrow) made on the stump. 20 min after operation.
14: The same, 65 min after operation.
15: The same, 90 min after operation.
16: The same, 150 min after operation.

Changes of structure during proboscis regrowth.

17: A transverse section through the distal part of proboscis. The continuous line links right and left paracytostomal kineties, thus dividing oral from somatic parts of proboscis. Central fiber (cf.). × 5 500
18: A transverse section through the proximal part of proboscis. The continuous line links right and left paracytostomal kineties, thus dividing oral and somatic parts. Central fiber (cf.). × 5 000
19: The resorbed cilia (arrows) in the cytoplasm of regrowing proboscis 15–25 min after operation. On the left side oral kinetosomes are visible. × 8 500
20: A section through the cytostomal field (cs.f.) and a somatic region with somatic kinetosomes (s.k.). 40–60 min after operation. The arrow indicates the resorbed cilium. No sections of cilia are visible in the area between the cytostomal field and the somatic region. × 11 500
21: Arrangement of ciliated kinetosomes around the cytostomal field (cs.f.). 30–60 min after the operation. Stained with osmium. Arrows indicate lack of curved segments of somatic kineties close to the cytostomal field. × 100 obj.

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22: A section through the cytostomal field (cs.f.) and the somatic region with the somatic kineties (s.k.). 15–25 min after the operation. Arrows indicate resorbed cilia. Continuous line indicates the course of the circumcytostomal kinety. Discontinuous lines indicate the course of the somatic kineties. × 14 500
23: The pattern of oral and somatic ciliature in the regrowing proboscis, 0–30 min after operation. Cytostomal field (cs.f.). Arrows indicate the circumcytostomal ciliature. Preparation stained with protargol. × 10 obj.
24: Somatic region of the body close to the cytostomal field (cs.f.). Arrows indicate some somatic kineties on the right side of the cell. Dense kinety (d.k.). The differences of density of kinetosomes in these kineties are visible. Preparation stained with protargol. × 100 obj.
25 a: A section through the circumcytostomal kinety 40–60 min after the operation. Arrows indicate some new oral kinetosomes. Resorbed cillium (r.c.), nemadesma of external basket (nd.), transverse fiber (t). × 11 500
25 b: The formation of new oral kinetosomes. × 22 000
26: A picture illustrating the folding of the oral kinety (arrows) on one side of the cytostomal field (cs.f.) 30–60 min after operation. Preparation stained with protargol. × 100 obj.
27: Base of the proboscis. A section through the right paracytostomal kinety (r.pc.k.) and dense kinety (d.k.). Discontinuous lines show courses of somatic and oral kineties. Folding of these kineties is visible. 100–120 min after operation. × 19 500
28: The oral kineties on the right side of the cytostomal field (cs.f.) 30–60 min after operation. Arrows indicate folding on the base of proboscis. Preparation stained with protargol. × 100 obj.

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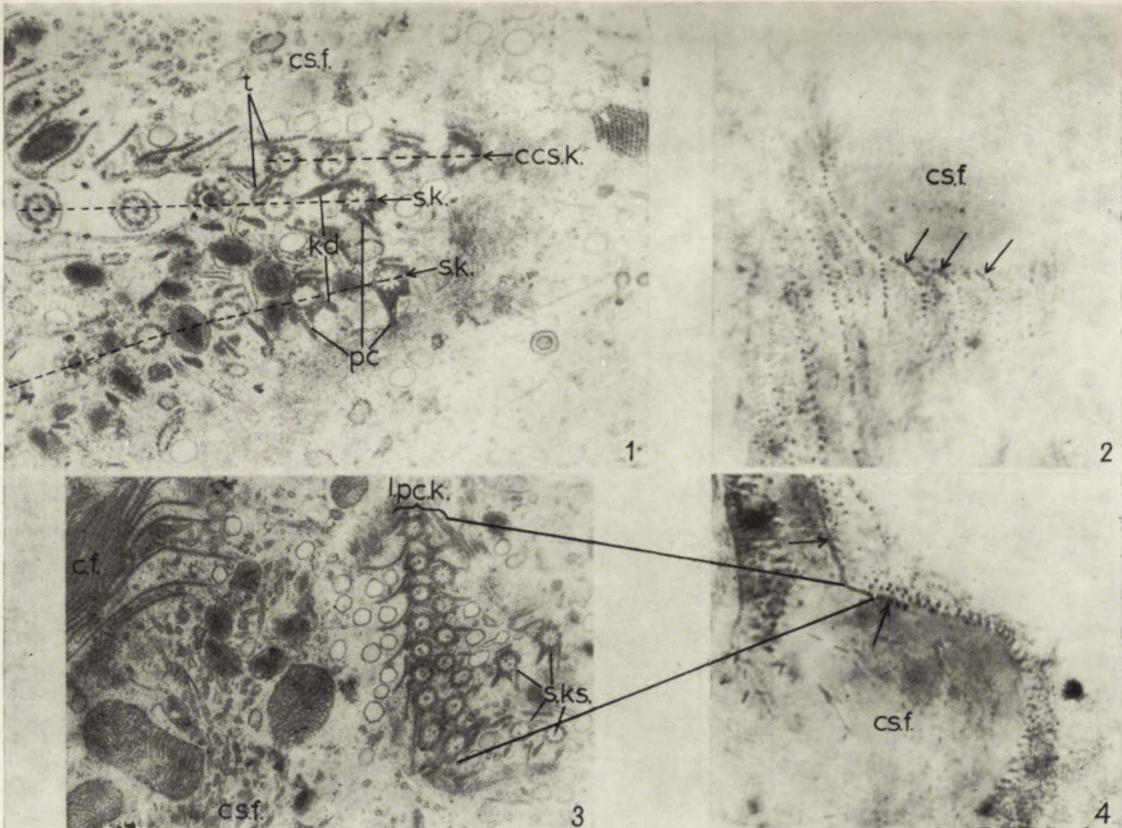

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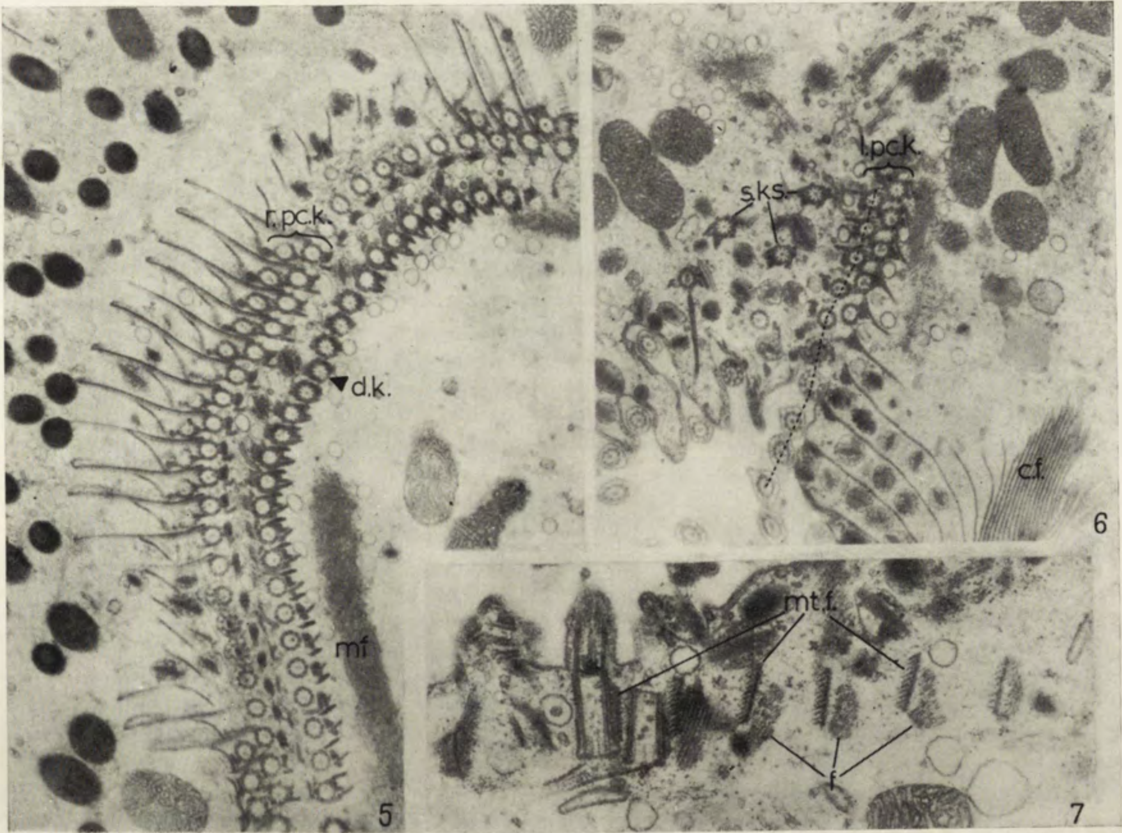

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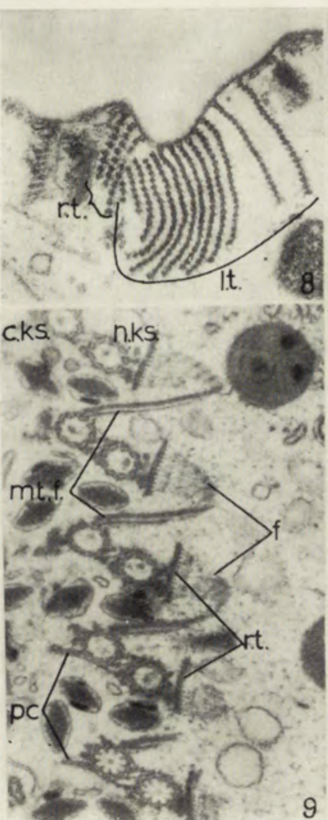

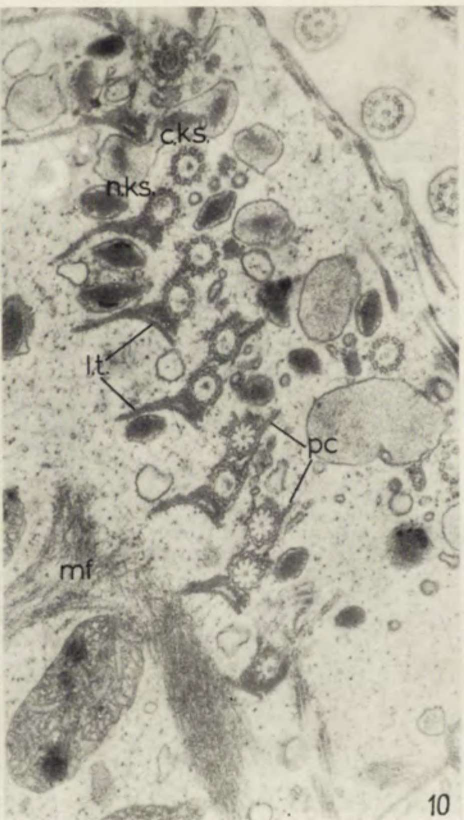

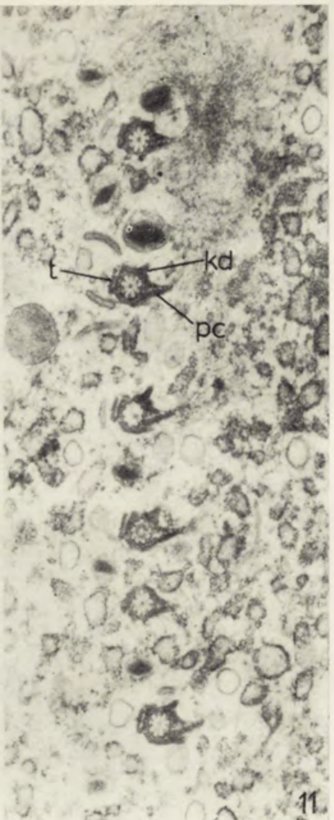

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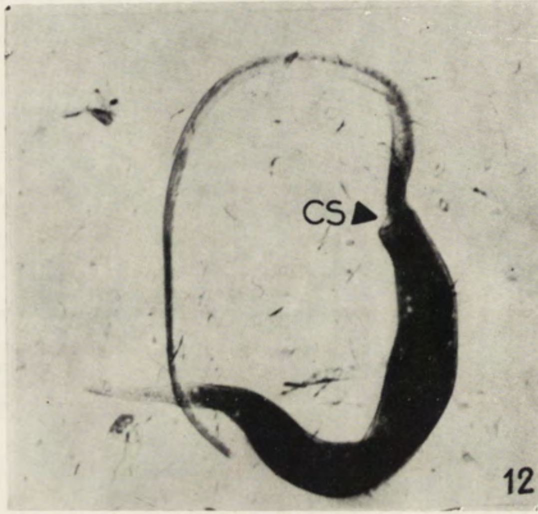

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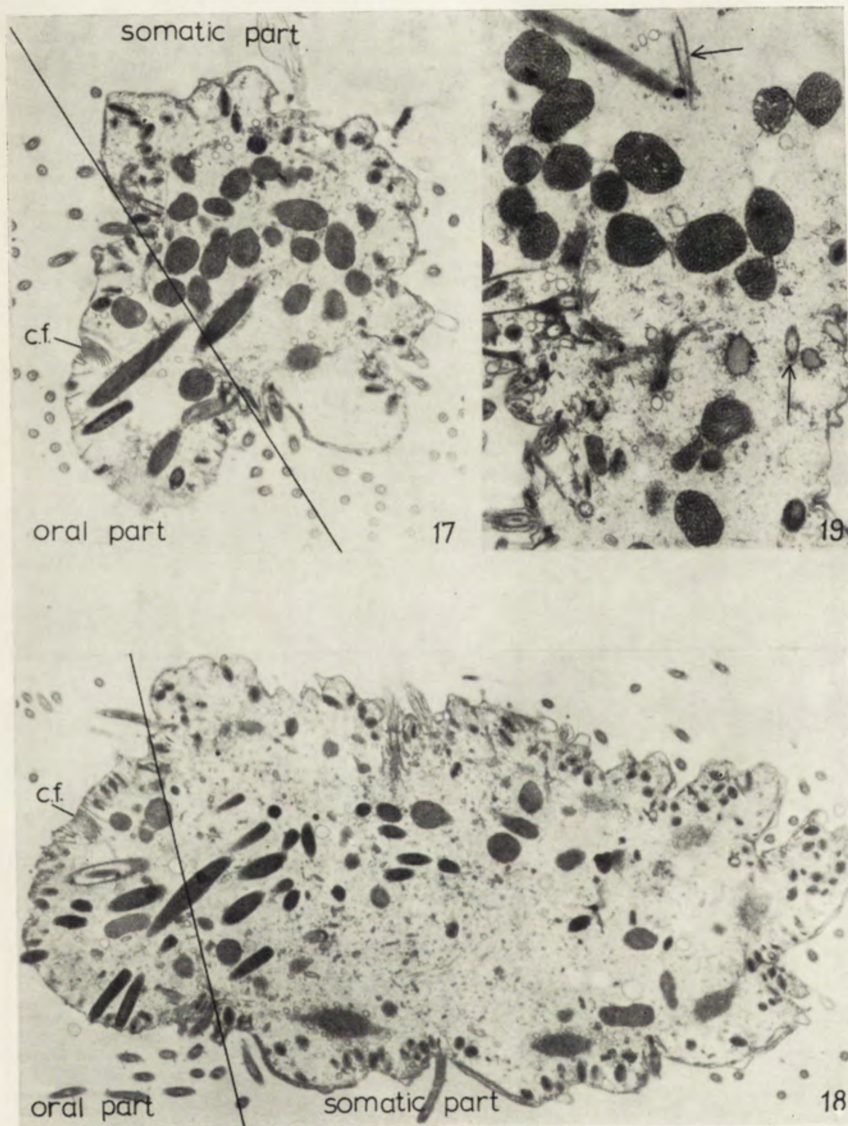

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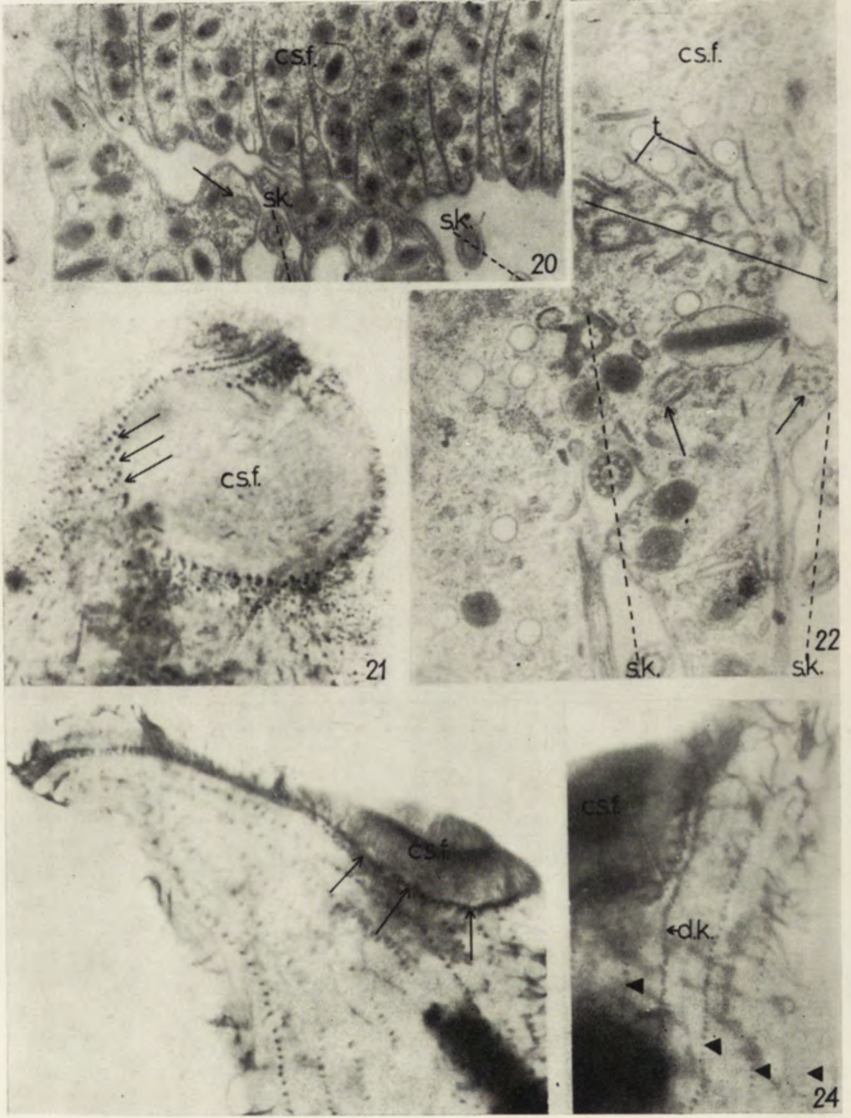

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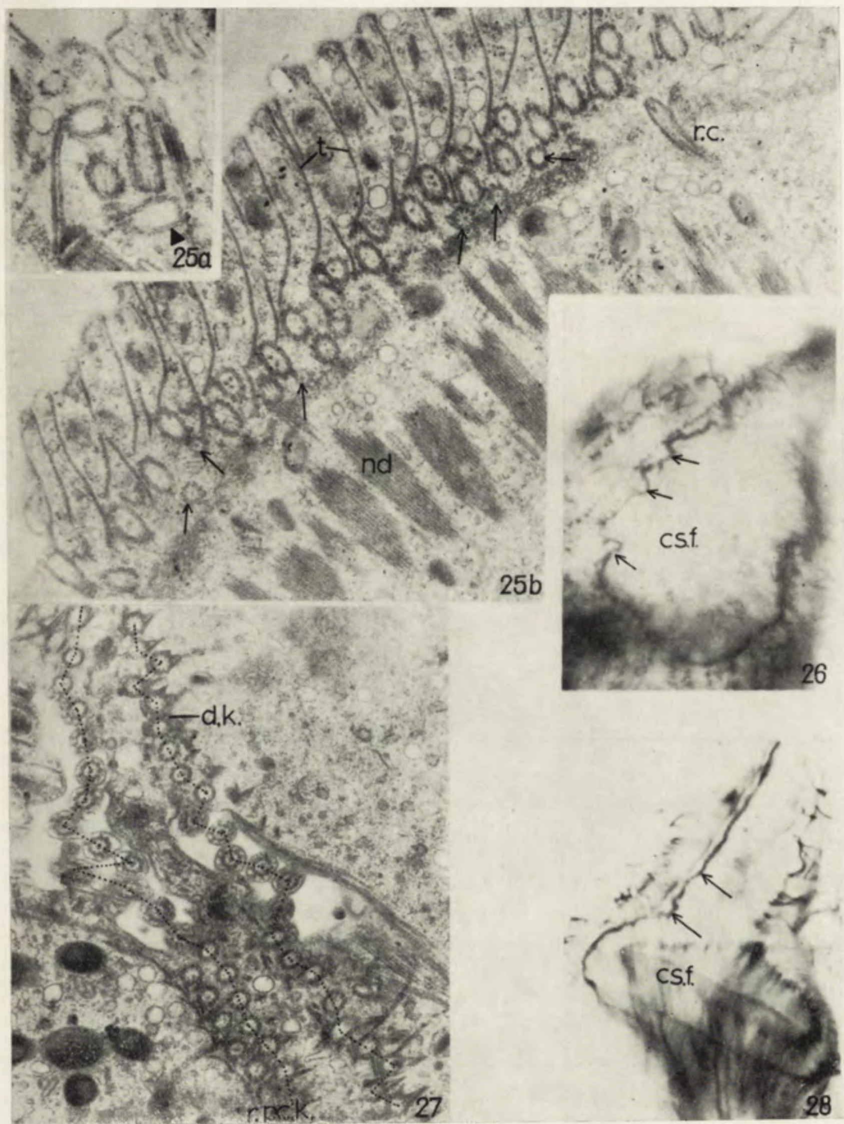

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Variations in Carbohydrates, Glycogen and Proteins in *Blepharisma intermedium* During Synchronous Division

Synopsis. Synchronous division in *Blepharisma intermedium* was induced by temperature shifts — 12°C for 95 h and 38°C for 24 h. Quantitative differences in carbohydrates, glycogen and proteins of *Blepharisma intermedium* exposed to 12°C for 95 h, 38°C for 24 h have been studied and compared with the ciliates grown at room temperature.

The life cycle of an animal cell is characterized by a well coordinated synthesis of various macromolecules leading to an increase in cell mass, replication of genetic material and ultimately a sharing of macromolecules as a result of mitosis (Prescott 1964). Until recently, most of the information concerning these events was obtained by observing single cells in randomly dividing cultures (Feinendegen and Bond 1963, Killander and Zetterberg 1965, Konrad 1963, Prescott and Bender 1962, Prescott 1964, Scopes and Williamson 1964, Taylor 1960). However, detailed quantitative investigations at the biochemical level require material in amounts far exceeding that which one cell can supply. This has led to a demand for cultures in which the cells engage simultaneously in division. This is called synchronous division. Synchronous division of *Blepharisma intermedium* was obtained by temperature treatment. Biochemical assay of total carbohydrates, glycogen and proteins were made on synchronously dividing *Blepharisma intermedium.*

Methods

Pure cultures of *Blepharisma intermedium* Bhandary grown in hay infusion fortified with horlicks was exposed to temperature shocks. The four methods used to induce synchronous division by temperature shocks have been described in an earlier paper (Kasturi Bai and Tara 1972).

As the synchronous division was maximum in the cultures grown at 12°C for 95 h and at 38°C for 24 h, biochemical investigations were made on *Blepharisma*

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intermedium acclimated to 12°C for 95 h and 38°C for 24 h at the end of the heat shock. Investigations were made on ciliates acclimated only to the cold 12°C for 95 h and compared with the ciliates grown at room temperature 26°C.

Carbohydrates

The assay for total carbohydrates was adapted from Dubois et al. method (1956). 100,000 ciliates were centrifuged and the pellet was washed three times in 0.15 M KCl and thrice in Dryl's solution (1959). This pellet was kept at 60°C until constant weight was obtained. Then pellet was washed in 1 ml of 1 N NaOH and then made up to 10 ml, 0.1 to 0.2 ml of this was diluted to 1 ml and 0.05 ml aliquot of 80% phenol was added. Five ml of concentrated H_2SO_4 were then added and the optical density was determined at 490 nm after 30 min incubation at 25–30°C.

Glycogen

The method adapted for glycogen assay was from McCready et al. (1950) and Cook (1967). About 40,000 ciliates were centrifuged and collected into a pellet. They were washed three times in 0.15 M KCl and thrice in Dryl's solution (1959). The washed pellet was extracted with boiling 55% ethanol until the supernatant did not give colour for the presence of sugars with Anthrone reagent. This cell residue was hydrolysed at 25°C for at least 10 min in 2 ml of 1 N NaOH. Two ml of 50% perchloric acid were added to the hydrolysate which was then chilled to 0–5°C. About 0.5 ml to 1 ml was used for development of colour to a final volume of 2 ml, 10 ml of 0.1% Anthrone reagent (1 g/72% H_2SO_4) was used and the sample was heated in a boiling water bath for 10 min, cooled to room temperature and the optical density was read at 630 nm.

Proteins

For protein analysis, about 40,000 ciliates were centrifuged and collected into a pellet. They were washed three times in 0.15 M KCl and three times in Dryl's solutions (1959). The washed pellet was resuspended in 5% trichloroacetic acid for 20 min at room temperature. The acid insoluble material was washed twice in 5% TCA and then total protein determinations were made on the final pellet by the method of Lowry et al. (1951) and the optical density was determined at a wavelength of 660 nm.

Results

Quantitative estimations of total carbohydrates, glycogen and proteins revealed one thing in common. The amount of all these substances was more in *Blepharisma intermedium* grown at 12 °C for 95 h and exposed to 38 °C for 24 h when compared to the ciliates grown at room temperature as well as ciliates acclimated to only 12 °C for 95 h. The results are summarized in Table 1.

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Table 1

Blepharisma intermedium exposed to cold and warm temperature — 10^6 cells

	Normal	Cold 12°C 95 h	Hot 38°C 24 h, after cold exposure (12°C for 95 h)
Proteins	61 mg	59 mg	93 mg
Glycogen	11 mg	9 mg	12 mg
Carbohydrates	14 mg	20 mg	31 mg

Discussion

The manner in which the biosynthesis of cell components is regulated has been the subject of renewed interest in recent years. Our information has been largely derived from exponentially growing cultures. Such cultures contain a mixture of cells at various stages in the division cycle and their analysis yields average values of the individual members of the population. Hence, there is little information of the way in which these processes are integrated into the overall process of cellular growth and division in individual cells. With the exception of a few enzymes, techniques are not available for examination of the biosynthesis of macromolecules in single cells. The use of synchronously dividing cultures of microorganisms provides a means by which the events occurring in single cells can be amplified and therefore subjected to analysis by the usual biochemical tools. Much knowledge about the course of cellular changes during a cell generation cycle has been obtained from studies on single cells of known ages, with the aid of refined cytological techniques. For quantitative information on the changes in the cellular components, biochemical estimations of total carbohydrates, glycogen and proteins were made.

Metabolic studies on the availability of energy for cell division, first made on dividing sea urchin eggs, have shown that aerobic conditions along with efficient respiration and carbohydrate utilization are necessary for mitosis (Krahl 1950, Swann 1957). On the basis of these studies the theory originated that the energy for division must be built up before the cell enters mitotic prophase (Bullough 1952, Brachet 1957, Swann 1957). This theory was extended and elaborated by Bullough in his reports on mouse ear epidermis cultured *in vitro* (1952, 1955). Bullough has tried to show that controlling mechanism for mitosis lies in the accumulation of energy from glucose during a "critical" period prior to visible prophase that he has called antephase.

The presence of carbohydrates, fats and proteins in protozoa are con-

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sidered as reserve food material. The accelerated rate of respiration (Curnutt and Schmidt 1964) synthesis of RNA and DNA (Schmidt and Herrmann 1965) and deposition of cell wall etc., during nuclear division in the high temperature strain of *Chlorella pyrenoidosa*, when the rate of photosynthesis was at a minimum (Sorokin 1957) suggested that a previously accumulated carbohydrate reserve was mobilized during this event to supply the carbon precursors and energy for organic synthesis. Accumulation of glycogen in response to the heat shocks was demonstrated by Scherbaum and Levy (1961). The accumulation seems to be faster than other macromolecules during the synchronizing treatment. Accumulation of more complex carbohydrates, some rich in amino sugars has been reported by Lindh and Christensson (1962) and Christensson (1962). These authors deal extensively with aggregates of carbohydrates and RNA. Accumulation of carbohydrates or energy reserves in one period will free the cells labours for increased respiration and a generally increased synthetic activity in the next period (Padilla et al. 1966).

The results of the present investigation show an increase in the total carbohydrates and glycogen after temperature treatment (Table 1). However, there is no wide variation in the amount of glycogen content at the three temperatures studied. There is considerable difference in the amount of total carbohydrates present. At the end of the heat shock it is nearly double the quantity present at 26 °C.

Zhinkin (1929) notes that in *Stentor* glycogen is accumulated at low temperatures and consumed at high temperatures. In this investigation, the quantity of glycogen present in *Blepharisma intermedium* during cold temperature (12 °C) is less than in the warm temperature (38 °C).

In synchronous cultures of yeast, the increase in mass, protein and RNA continue approximately linearly throughout the division cycle, with the rate of synthesis doubling with each generation (Gorman et al. 1964, Mitchison 1957, Mitchison and Cummins 1964, Scopes and Williamson 1964, Tauro 1965). The total cellular content of protein, RNA and DNA and photosynthetic pigments has been measured in synchronized *Euglena gracilis* (Cook 1965). Protein and RNA here are synthesized in parallel in synchronized *Euglena*. This synthesis continues in a linear manner over the entire 24 h light-dark cycles when expressed per millilitre of culture (Cook 1965). In *Tetrahymena pyriformis* (Padilla et al. 1966), biochemical measurements of cellular content of protein, RNA, DNA and carbohydrates were made on synchronized cells. The results suggested that on increase in DNA templates in one period will allow increased synthesis of RNA in the

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next period. Increased RNA would in turn allow more protein synthesis in the next period. The protein content of *Tetrahymena pyriformis* varies with the culture conditions. In exponentially multiplying populations, values from 0.74 to 3.2 mg protein per million cells have been published (Christensson 1959, Lee et al. 1959, Leick 1967, Scherbaum and Levy 1961, Singer and Eiler 1960). The first attempt to measure differential increases in the cell proteins during the period of heat shocks was made by Christensson (1959). Throughout the synchronizing heat treatment a general increase in protein content has been observed (Byfield and Scherbaum 1966, Christensson 1959, Lee et al. 1959, Leick 1967, Scherbaum et al. 1959). Changes in the protein composition before and during cell division have been demonstrated by Watanabe and Ikeda (1965 b, c). Very little information is available on the effect of a single heat shock in the synchronizing programme on the synthesis of various fractions of cell protein. However, there are few reports dealing with the rate at which total protein is synthesized during the heat treatment (Mita et al. 1959, Watanabe and Ikeda 1965 a). Hamburger and Zeuthen (1960) have found an increase in the protein content in *Tetrahymena pyriformis* after the heat treatment.

In this investigation synchrony in division has been induced in *Blepharisma intermedium*, with only a single heat shock. It is found that there is considerable increase in the quantity of protein at the end of heat treatment. This is in agreement with Hamburger and Zeuthen's (1960) report. They have found a similar increase in the protein content of *Tetrahymena pyriformis* at the end of the heat treatment.

Reserves of glycogen, carbohydrates and proteins increase in quantity at the end of the heat treatment in synchronized cells of *Blepharisma intermedium*.

RÉSUMÉ

La division synchronique du *Blepharisma intermedium* a été obtenue par changements de température —12°C pendant 95 heures et 38°C pendant 24 heures. La différence quantitative en hydrocarbones, gycogine et protéines du *Blepharisma intermedium* exposé à 12°C pendant 95 heures, comme celui exposé à —38°C pendant 24 heures a été étudiée et comparée avec les "ciliates" développés en chambre (température normal).

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Cytoplasmic Streaming within *Paramecium aurelia*. IV. Cyclosis During Binary Fission and Conjugation

Synopsis. Cyclosis shown by the *Paramecium aurelia* is ceased at the time of completion of binary fission and at the presumable exchange of migratory pronuclei in conjugation. In both cases cyclotic streaming of cytoplasm arises soon again, either in fission products and in conjugating pair, after completion of mentioned above stages.

Our previous papers (Kuźnicki and Sikora 1971, 1972, Kuźnicki et al. 1972) deal with the main features of cytoplasmic streaming within *Paramecium aurelia*. Variety of agents can disturb or cease the cyclotic activity of cytoplasm (Koenuma 1954, 1963, Yamashita 1964, Yamada 1969, 1970, 1974 a, b, Kuźnicki and Sikora 1973 a, b). The evidences reported by Kuźnicki et al. (1972) suggest that cytoplasmic streaming might be also ceased and arisen again without influence of external stimuli what means that the cell is able to control the movement of its cytoplasm. From the other hand careful observation shows that activity of cytoplasm seems to be dependent in some extent of the phase of conjugation (Wichterman 1940, Sikora and Kuźnicki 1973). Therefore the aim of the present paper was to examine the cases when cessation of cytoplasmic streaming seems to be related with the some events of the life cycle of *Paramecium aurelia* namely: interfission, binary fission and conjugation.

Material and Methods

The cillate studied was *Paramecium aurelia* stock 51, syngen 4, serotype 51A. It was cultured in lettuce infusion inoculated with *Aerobacter aerogenes* at 27°C (Kuźnicki and Sikora 1971). Food was provided to obtain interfission period about 7–8 h.

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Early dividers of *P. aurelia* have been obtained by means of "method of thress" (Ehret and DeHaller 1963) while mating reactive ciliates have been obtained following Sonneborn's (1950, 1970) methods. Procedure for immobilization applied was diluted antiserum method (Kuźnicki and Sikora 1971). The specimens which initially has not shown cyclosis were not taken into further experimental procedure. Records of observation were collected either by use of cinematography or long time exposure photography under polarizing microscope. Some observation were provided on thigmotacticly settled ciliates. Dippell (1955) staining was used to check the stages of conjugation at the nuclear level.

Results

Judging by the appearance of the pattern and the velocity of cytoplasmic streaming within different length interfission paramecia (Pl. I 1, Fig. 1 a) it was not possible to distinguish significant differences among them. Careful examination of more than 25 specimens of antiserum immobilized paramecia at different interfission stages between subsequent divisions has shown that only short lastig cessation of cytoplasmic streaming occur (Kuźnicki et al. 1972). It seems that interfission paramecia are able to exhibit the cyclotic movements of cytoplasm throughout the whole period.

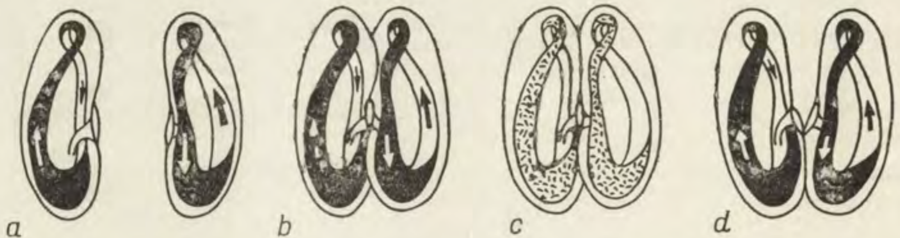

Fig. 1. These drawings show the schematic pattern of cyclosis in the subsequent stages of conjugation of *Paramecium aurelia*: before conjugation (a), within conjugating pair when cyclosis is still present (b), the cessation of cyclosis (c), and when cyclosis appear at the last stages of conjugation (d)

Every pair of 24 studied conjugating paramecia, either antiserum immobilized, or thigmotacticly settled down, exhibit vigorous cytoplasmic streaming (Pl. I 2, Fig. 1 b) except the stage shown on the Pl. I 3, Fig. 1 c. The cessation of cytoplasmic streaming occurs at the time related to the third division of micronuclei and probably migratory nuclei exchange, judging from the Dippell (1955) staining preparations. Before separation of conjugating paramecia, the cyclotic activity arises again (Pl. I 4, Fig. 1 d).

It have been proved that the progress of binary fission of *P. aurelia*

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immobilized by means of antiserum is not affected. By use the "method of thress" (Ehret and DeHaller 1963) predivisional cells were collected and immobilized. At a few minute intervals succeeding stages of division was determined upon the appearance of fission furrow on the left side of the cell and photographic records were taken. As it is shown on the long time exposure photography (Pl. II 5, 6, Fig. 2 a, b, c) at stages when the first signs of the furrow arise and are in progress the cytoplasmic streaming has its normal velocity and pattern. Just before the moment of furrow completion (Pl. II 7) in every one examined case cytoplasmic streaming ceases (Pl. II 8, Fig. 2 d). After dividing off into

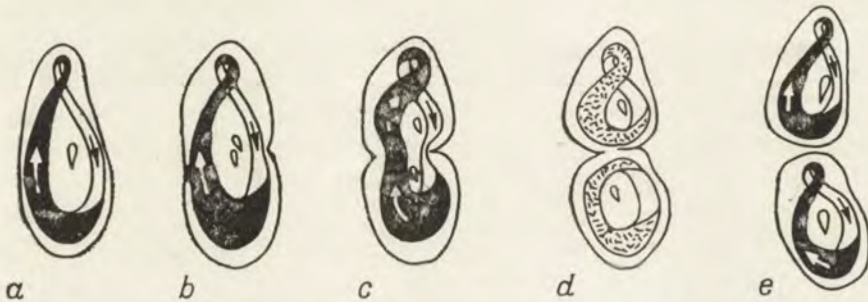

Fig. 2. These drawings show the schematic pattern of cyclosis in the subsequent stages of binary fission of *Paramecium aurelia*: (ventral side facing to the viewer): interfission specimen (a), predivisional stages (b, c), just before completion of fission when cyclosis is ceased (d), two products of binary fission with cyclosis (e)

daughter cells the cytoplasmic streaming appears again (Pl. 9, 10, Fig. 2 e). The processes of cessation and reappearance of cytoplasmic streaming in conjugating members and in dividing specimen of *P. aurelia* are generaly much alike in both phenomena. Completion of cessation is preceded by increasing retardation of the velocity of whole streaming along the route. After few (3–6) min only flickering crystals are seen eventualy entirely ceased. Reappearance of cyclosis after completion of presumed exchange of micronuclei in conjugation and completion of cleavage of dividing cell is preceded by increase of vibration of particles at the area when cytoplasm flows in cyclosis. The first signs of the local narrow streamings appear mainly at the base of dorsal branch of cyclotic route (Fig. 1 d), however, it may often disappear for some time. Transition between motionless state and complete renormalization of the pattern of cyclosis is not sharp therefore timing of these processes is difficult. Rough estimation shows that processes of renormalization of cyclosis take place in case of conjugation about 30–40 min and in case of binary fission about 20 min what is significantly slower when compared with the rate of short lasting cessation of cyclosis.

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Discussion

Due to the point in question when the cytoplasmic streaming might be stopped in relation to the stage of the life cycle it was proved that the short lasting cessation (Kuźnicki et al. 1972) might take place throughout the whole interfission period while long lasting cessation occur at the time of completion of binary fission and at the time of presumable exchange of migratory pronuclei in conjugation (Sikora and Kuźnicki 1973). It is likly that the autogamous *Paramecium aurelia* at the stage related to the conjugation also shows cessation of cytoplasmic streaming, however, it was not possible to prove that yet. The main obstacle was in timing of the nuclear events concomitantly with the cytoplasmic activity observation. In the case of use of the phase contrast optics for visualization of the state of nuclei leads to flattening (Hanson et al. 1969, Hanson 1974), though distorting the normal shape of the cell what, however, improves the viewing of the specimen but affects it so much that cytoplasmic streaming is stopped (Sikora unpublished).

At the time of pronuclei exchange between conjugating *P. aurelia* Jurand (personal communication) had observed on the EM photographs the microtubules and microfilaments lying closely to the migratory pronucleus what seems to show the motive role of those structures. Concomitantly at the same stage the cytoplasmic streaming is ceased. Therefore it might be presumed that long lasting cessation of cytoplasmic streaming occurs in those stages in life cycle when there is need of direct and precise location of the cell components performed by other motive systems than those promoting cytoplasmic streaming. Otherwise micronuclei division products might be randomly "carried by currents away" (Diller 1936). There is no reasonable explanation why cyclotic activity of cytoplasm is ceased at the time of completion of binary fission. It should be pointed out, that long lasting cessation might be due to the influence of environmental agents probably at any time of life cycle. This aspect of cytoplasmic streaming behaviour will be under consideration elsewhere.

RÉSUMÉ

La cyclose chez *Paramecium aurelia* est interrompue pendant le stade de la division cellulaire, et pendant la période de la conjugaison quand probablement s'effectue l'échange des pronoyaux migratoires. Dans les deux cas le courant cytoplasmique circulaire réapparaît aussitôt après l'accomplissement de ces stades, aussi bien dans les cellules produites par la division que chez les partenaires en conjugaison.

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EXPLANATION OF PLATES I-II

1–10: Long time exposure photographs under polarizing microscope of antiserum immobilized *Paramecium aurelia*. Birefringent crystals are see as a white dots or paths when are flowing with the cytoplasmic streaming

1: Interfission specimen with the normal pattern of cytoplasmic streaming. Exposure time 30 s

2: Early stage of conjugation. Arrows points the direction of the flow of cyclic streaming within both mates. Exposure time 15 s

3: Cessation of cytoplasmic streaming at the stage of third division of micronuclei in conjugating pair. Exposure time 15 s

4: The stage of conjugation just before separation of mates. Cytoplasmic streaming arise again. Arrows indicates vigorously flowing birefringent crystals. Exposure time 30 s

5–10: Subsequent stages of the binary fission of the same specimen of *Paramecium aurelia* (dorsal side faceing to the viewer)

5: Early stage of binary fission when cytoplasmic streaming has still its normal pattern. Exposure time 10 s

6: Binary fission in progress. Velocity of cytoplasmic streaming is reduced. Exposure time 15 s

7: Cessation of cytoplasmic streaming just before completion of division furrow. Exposure time 10 s

8: Completion of division furrow. No signs of cytoplasmic streaming. Exposure time 15 s

9: First signs of cytoplasmic streaming indicated by the arrows. The cells remain attached to each other because both are immobilized. Exposure time 10 s

10: Recovery of the normal pattern of cytoplasmic streaming within proter and opisthe. Only in opisthe the circular character of the streaming is well seen, while in proter the turn along the long axis of the cell occur and only facing part of streaming is in the focus. Exposure time 10 s

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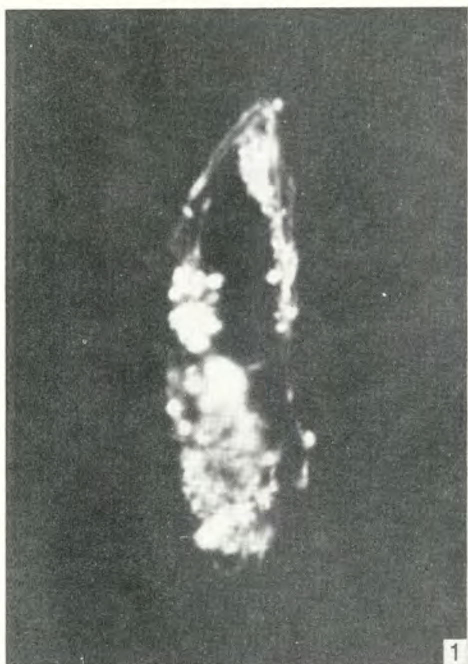

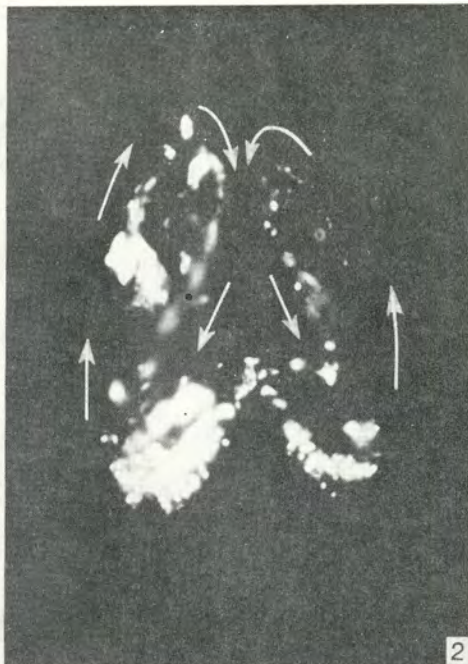

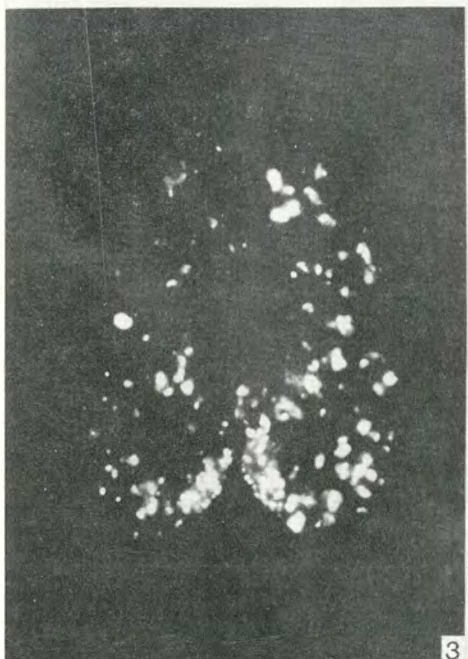

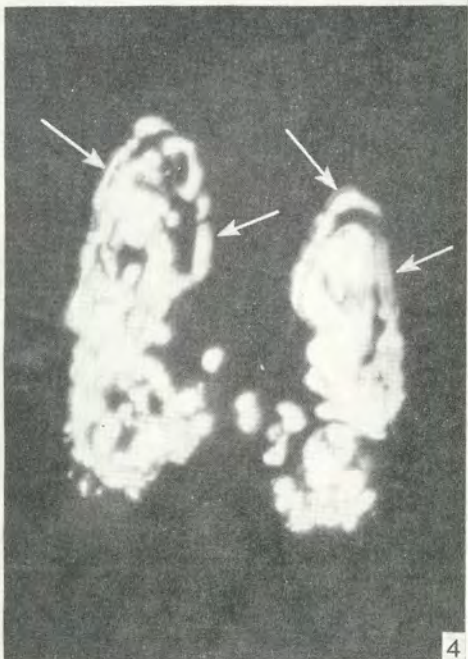

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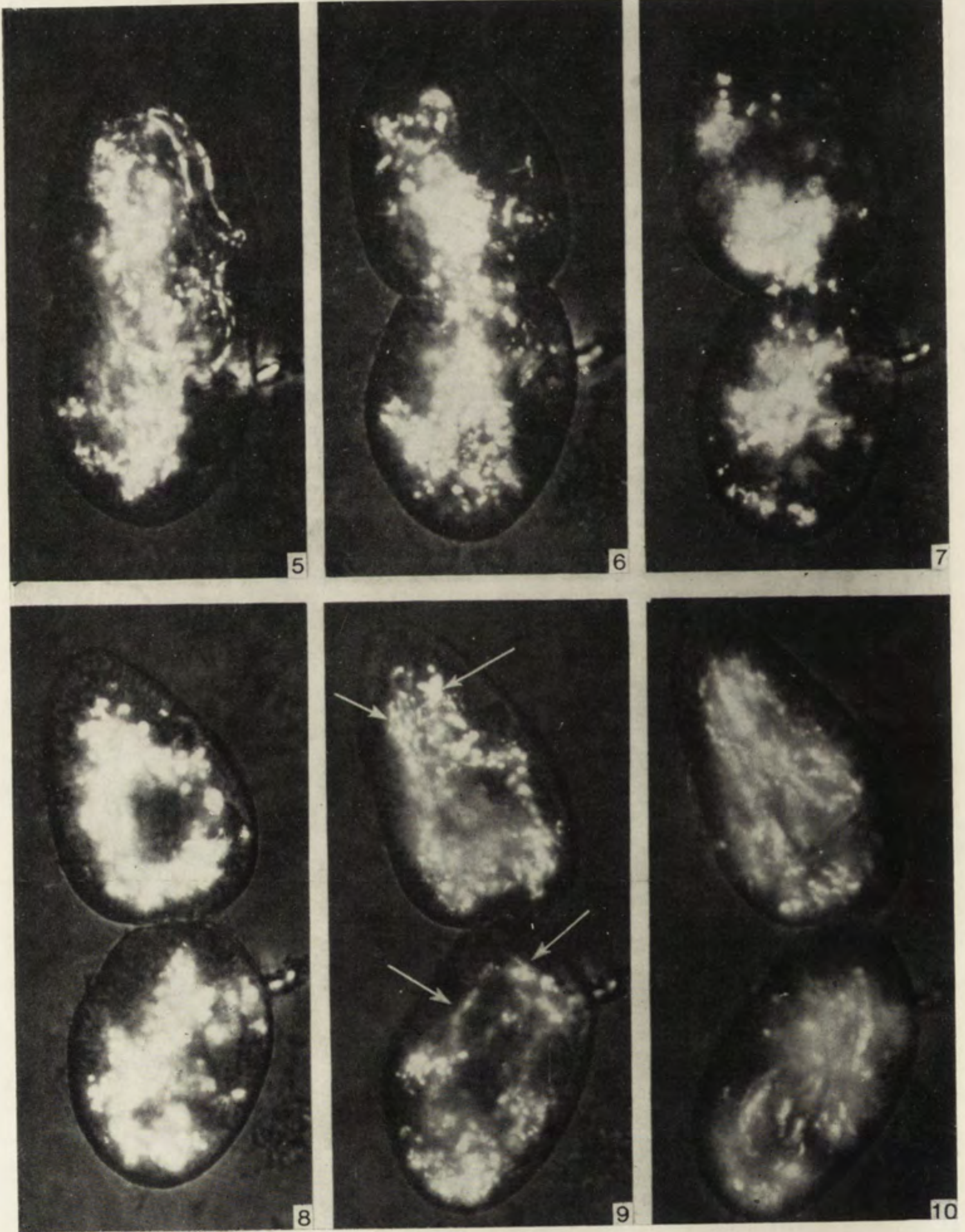

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Two Types of Hyaline Caps, Constricting Rings and the Significance of Contact for the Locomotion of *Amoeba proteus*[2]

Synopsis. The renormalization of locomotion in specimens of *Amoeba proteus* originally showing fountain-like cytoplasmic streaming is accompanied by typical changes in the morphology of the hyaline cap. The hemispherical shape of the cap in amoebae exhibiting fountain-like streaming results from the pressure exerted upon it by the streaming endoplasm. This can be followed by the observation that the cap assumes a spherical shape when the cytoplasmic streaming changes direction and the pressure is decreased. It is transformed then to a normal cytoplasmic vacuole with a watery content comparable to that of the pulsating vacuole. Constricting rings travelling along the periphery of incompletely attached specimen on siliconized glass could be followed in detail. The behaviour of these rings suggests that they result from ectoplasmic contractions. Changes in the polarization of structure and motile activities, induced by local attachment of the cell surface, were observed. These phenomena were interpreted as further evidence for a significant role of the cell membrane in motile behaviour in amoebae.

The significance of hyaline caps for the locomotion of large amoebae of the *proteus-chaos*-group has been discussed by several investigators (Mast 1926, Goldacre 1958, 1961, Allen 1962, Kavanau 1965). According to recent observations on *Amoeba proteus* (Korohoda and Stockem, 1975), two distinct types of hyaline caps differing in structure and function could be distinguished. In pseudopodia extending during normal locomotion the hyaline cap consisted of pure

[1] This work was carried out during the year Dr. Korohoda spent in the Institute of Cytology and Micromorphology in Bonn. The author wishes to thank Prof. dr. K. E. Wohlfarth-Bottermann for hospitality and the Alexander von Humboldt-Stiftung for a scholarship.

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[2] The authors are indebted to Prof. dr. J. Abel for reading and discussing the manuscript.

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ground cytoplasm, whereas in specimens showing fountain-like streaming it contained a watery fluid with a low content of organic substances. In the present communication further evidence is given that this type of hyaline cap represents a cytoplasmic vacuole. Besides this some observations on the existance of contractile rings and the contact of the cell surface of the amoebae with the glass as well as the functional polarization in the morphology and activity of the cells are reported.

Material and Methods

Amoeba proteus (Princenton strain) was grown according to the method described by Haberey and Stockem (1971). All observations were carried out be means of Zeiss photomicroscope (Zeiss, Oberkochen) equipped with dark field, normal phase contrast or differential interference contrast optics.

For scanning electron microscopy the amoebae were rapidly frozen in liquid freon, freeze-dried in a Leybold apparatus at −160°C for several days, coated with gold and photographed in a Cambridge S_4 stereoscan electron microscope [3].

Results

(1) Hyaline cap-vacuole

Seravin (1966) reported that monopodial amoebae started normal locomotion when he separated or punctured the hyaline cap. We observed the same effect after transferring specimens showing fountain-like streaming (Pl. I 2 and II 4) to new culture medium containing Chalkley-solution with $CaCl_2$ up to a final concentration of 1 mM. A sequence of changes in morphology of the hyaline cap during renormalization of locomotion in *Amoeba proteus* is shown in Pl. II 5–10, III 11–14. Immediately after reversal of cytoplasmic streaming (Pl. II 5–7, III 11–13) the hyaline cap became separated from the cell membrane by a thin layer of ectoplasm (Pl. II 8, III 13 and 14, arrow-head). At the same time characteristic changes in shape of the hyaline cap occur. The cap which is normally flattened at its posterior border (Pl. II 4, 5–7, Pl. III 11 and 12) rounds up (Pl. III 13) and becomes completely spherical (Pl. II 8). While the amoeba gradually returns to normal locomotion by the formation of new pseudopodia (Pl. II 9 and 10), the spherical hyaline

[3] We have to thank Prof. dr. W. Kloft (Institute of Applied Zoology, University of Bonn) for the opportunity to use the stereoscan.

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cap is transported to the uroid region. Later the vacuole-like cap disappeared. We were unable to determine whether its hyaline content was resorbed by the cytoplasm or extruded from the cell through the cell membrane.

The hyaline cap during fountain-like streaming in *A. proteus* appears to represent a vacuole with a watery content. This can be concluded both from the above described results and the observation that a typical hyaline cap is always formed after a large cytoplasmic vacuole has been transported to the tip of a pseudopodium. This causes the cell to attain a monopodial form and the vacuole to assume a hemispherical shape by the pressure exerted upon it by the streaming endoplasm. Finally we were never able to follow cytoplasmic particles entering the hyaline cap fluid. These particles always flowed around the hyaline cap vacuole within the thin ectoplasmic layer separating the membrane of the vacuole and the plasmalemma (c.f. Pl. III 14, arrow-head).

(2) Constricting rings

Käppner (1961) described constricting rings running along monopodial *Chaos chaos* amoebae after treatment with a 1 mM ATP solution. Similar rings (furrows) were later observed by Kanno (1969) in some non-locomoting specimens of *Amoeba proteus* incompletely attached to the substratum. We noticed the occurrence of such rings in monopodial cells of *Amoeba proteus* which did not show active locomotion and in which a hyaline cap vacuole was lacking (Pl. I 3 and Pl. IV 15–18). These rings were formed at the anterior end of the cell (Pl. IV 15, *b* and *a*) and shifted backwards (follow *c*, *b* and *a* in Pl. IV 16–18) with an average speed of 2 to 5 $\mu m \times s^{-1}$. This value is in agreement with measurements of Kanno (1969) and corresponds to the normal locomotion speed of amoebae attached to the substratum. The formation and disappearance of constricting rings can also be observed over a period of several hours in cells placed on siliconized glass. The transfer of such specimens to clean glass results in the attachment of the amoebae to the glass surface and in the reorganization of normal locomotion.

(3) Cell contact

Attachment of a cell to a solid surface has always been considered as a necessary precondition for normal amoeboid locomotion (c.f. Mast 1929). Local contact of originally unattached cells also causes significant changes in morphology and moving activity of *Amoeba proteus* (Pl. V 19–22). This could be demonstrated in specimens kept for several hours in suspension. Those cells attain a radiose form by the production of long, branched pseudopodia (Pl. V 19). The building of such pseudopodia

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is brought about by disorganized cytoplasmic streaming, whilst their retraction is accompanied with a corrugation or krinkling of the cell surface (Pl. V 23, c.f. Korohoda 1970). When one of these pseudopodia is brought in contact with the solid surface of the substratum (Pl. V 19, arrow), spreading occurs in this cell region (Pl. V 20). Some minutes later all other pseudopodia not attached to the substratum are retracted (Pl. V 21) and the endoplasm flows into the attached, extending cell region (Pl. V 22 and 24).

Discussion

Observations carried out on specimens of *Amoeba proteus* which show fountain-like cytoplasmic streaming proved that the content of the hyaline cap vacuole consists of a watery fluid with a low degree of organic substances (c.f. also Korohoda and Stockem, in press). These observations confirm earlier suggestions of Allen (1961, 1962, 1973), who assumed that the hyaline cap in fountain-like streaming amoebae represents water extruded from the endoplasm. However, this water is surrounded by a membrane and cannot be regarded as a product of syneresis within the frontal zone of extending pseudopodia.

The hyaline cap vacuole itself seems to be responsible for the appearance of the typical fountain-like streaming pattern in *Amoeba proteus*. This conclusion is in accordance with the results of experiments carried out by Seravin (1966). This author demonstrated that the microsurgical removal of the cap causes renormalization of amoeboid locomotion.

The occurrence of two distinct types of hyaline caps in large amoebae, i.e., the hyaline cap vacuole in fountain-like streaming amoebae and the hyaline cap composed of pure groundplasm in normally extending pseudopodia (Korohoda and Stockem, 1975) can explain different misinterpretations in earlier investigation. Hence it seems to be necessary in further experiments to define precisely which type of hyaline cap is being referred to.

Observations on the appearance and behaviour of constriction rings in non-locomoting, monopodial amoebae incompletely attached to the substratum are consistent with result of studies carried out in other cell types. Similar constricting rings have been observed in cells dissociated from amphibian embryos (Holtfreter 1948) and in *Entamoeba hystolitica* (Kanno 1969). Likewise, the waves of surface foldings in locomoting fibroblasts start at the advancing edge of the cell and then travel backwards to the posterior cell region (Ambrose 1963, Abercrom-

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bie et al. 1970, Korohoda 1971). Constricting rings in *A. proteus* can only be observed in specimens which are not attached to the substratum. The dynamic behaviour of the constricting rings suggests that they result from contractile processes taking place near the cell membrane in the subjacent layer of cortical ectoplasm, i.e., within the cell surface complex.

Amoebae which are able to produce constricting rings are characterized by the existence of an ectoplasmic cylinder built up of granuloplasm. This ectoplasmic cylinder seems to be less gelled than in normally locomoting cells where it is responsible for the rigidity of the pseudopodia. It remains to be elucidated whether in large amoebae of the *proteus-chaos*-group changes in cell shape are primarily brought about by contractile processes occurring within this ectoplasmic cylinder or by the contraction of the cell surface complex consisting of the plasma membrane and a very thin layer of hyaline ectoplasm (c.f. Korohoda and Stockem, 1975). The activities of the cell surface complex have been assumed to be responsible for changes in cell shape and for locomotion processes in tissue culture cells (c.f. Ambrose 1972, Perdue 1973, Wessells et al. 1973). As the existence of this complex has been neglected in discussions on the mechanisms of locomotion in large amoebae, its functional significance should be considered in further investigations on this problem.

Observations on the effect of a localized contact between the surface of the amoeba and the glass seems to be an additional support for the theorie that the properties and activities of the plasma membrane can control the polarization and motile activities of amoebae (Braatz-Schade and Stockem 1972, Korohoda 1972). The importance of electrochemical phenomena at the cell surface for the activation of contractile structures within the cytoplasm and for the motive force generation of cellular locomotion has been discussed by several investigators (c.f. Komnick et al. 1973). Amoebae of the *chaos-proteus*-group can therefore be regarded as a suitable model to elucidate the physiological significance of such phenomena.

ZUSAMENFASSUNG

Die Umwandlung von *Amoeba proteus* mit fontänenähnlicher Cytoplasmaströmung in Zellen mit normaler polypodialer Fortbewegungsweise ist von typischen Veränderungen im Bereich der hyalinen Kappe begleitet. Die hemisphärische Form der hyalinen Kappe in Zellen mit Fontänenströmung stellt eine Folge des Druckes dar, der vom fließenden Endoplasma auf die Struktur der Kappe ausgeübt wird. Dies kann aus der Beobachtung geschlossen werden, daß die hyaline Kappe eine

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sphärische Form annimmt, wenn sich die Strömungsrichtung des Cytoplasmas ändert und der Druck nachläßt. Dieser Befund sowie die weitere Verfolgung des Schicksals dieser Struktur lassen den Schluß zu, daß die hyaline Kappe eine an die Pseudopodienspitze verlagerte und durch den Strömungsdruck des Endoplasmas an dieser Stelle fixierte normale cytoplasmatische Vakuole darstellt.

In Zellen mit Fontänenströmung konnte das Bewegungsverhalten von kontraktilen Ringen entlang der Amöben-Längsachse analysiert werden. Dazu wurden silikonisierte Glasobjektträger benutzt. Nach diesen Beobachtungen sind Kontraktionen im Ektoplasma für das Zustandekommen dieser Ringe verantwortlich.

Lokale Kontaktstellen zwischen der Zelloberfläche von *Amoeba proteus* und dem Substrat bewirken Veränderungen im morphologischen und bewegungsphysiologischen Organisationsmuster. Diese Tatsache kann als weiterer Hinweis dafür gelten, daß die Zellmembrane eine wichtige Rolle bei der Steuerung amöboider Bewegungsvorgänge spielt.

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EXPLANATION OF PLATES I–V

Hyaline caps and significance of contact for locomotion of *A. proteus*

1–3: Scanning electron micrographs of freeze-dried *A. proteus*, 1 — polypodial form, 2 — monopodial form, 3 — monopodial form with contractile (constricting) rings (arrows); 500 ×

4: Dark-field-photograph of *A. proteus* showing fountain-like streaming (arrows); 700 ×

5–10: Transformation of a monopodial *A. proteus* showing fountain-like streaming (5) to a polypodial form showing polypodial locomotion (10). Note translocation and morphological changes of the hyaline cap vacuole (black arrows). The white arrows indicate the cytoplasmic streaming direction; 300 ×

11–14: Morphological changes of the hyaline cap vacuole during renormalization of locomotion in an amoeba exhibiting fountain-like streaming. The cell membrane and the membrane of the hyaline cap vacuole are separated by a thin layer of ectoplasm (arrow-head in (14)). The arrows indicate to the direction of endoplasmic streaming; 1600 ×

15–18: Dislocation of contractile rings (arrows a–d) in a monopodial *A. proteus* showing fountain-like streaming on siliconized glass. Time interval between single micrographs 15^s; 500 ×

19–22: Changes in morphology of an *A. proteus* kept in suspension when making contact and attaching to the glass (arrow). The attached pseudopodium extends, the non-attached pseudopodia are retracted; 300 ×

23: Higher magnification of retracting pseudopodia; 700 ×

24: Higher magnification of extending pseudopodia; 700 ×

Abbreviations used: *hc* — hyaline cap vacuole; *u* — Uroid region.

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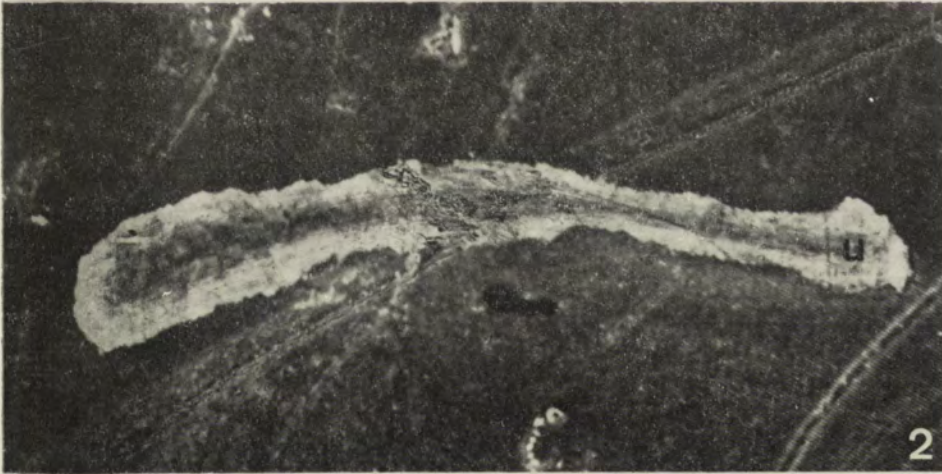

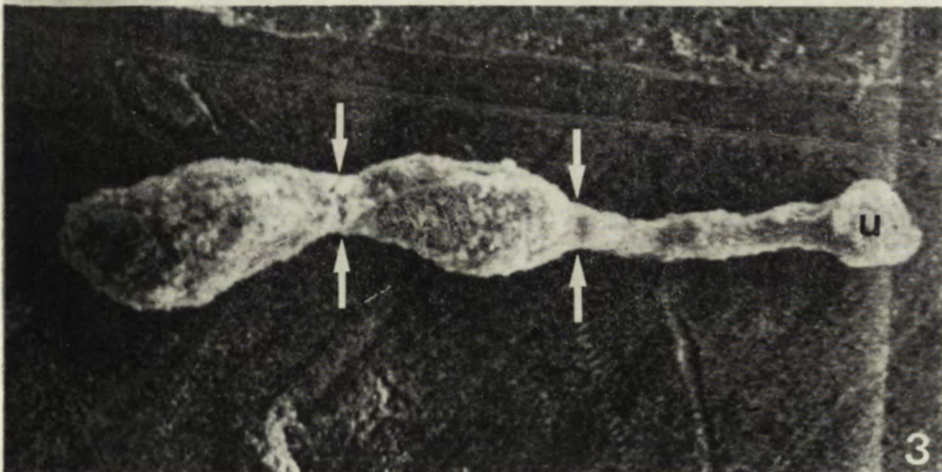

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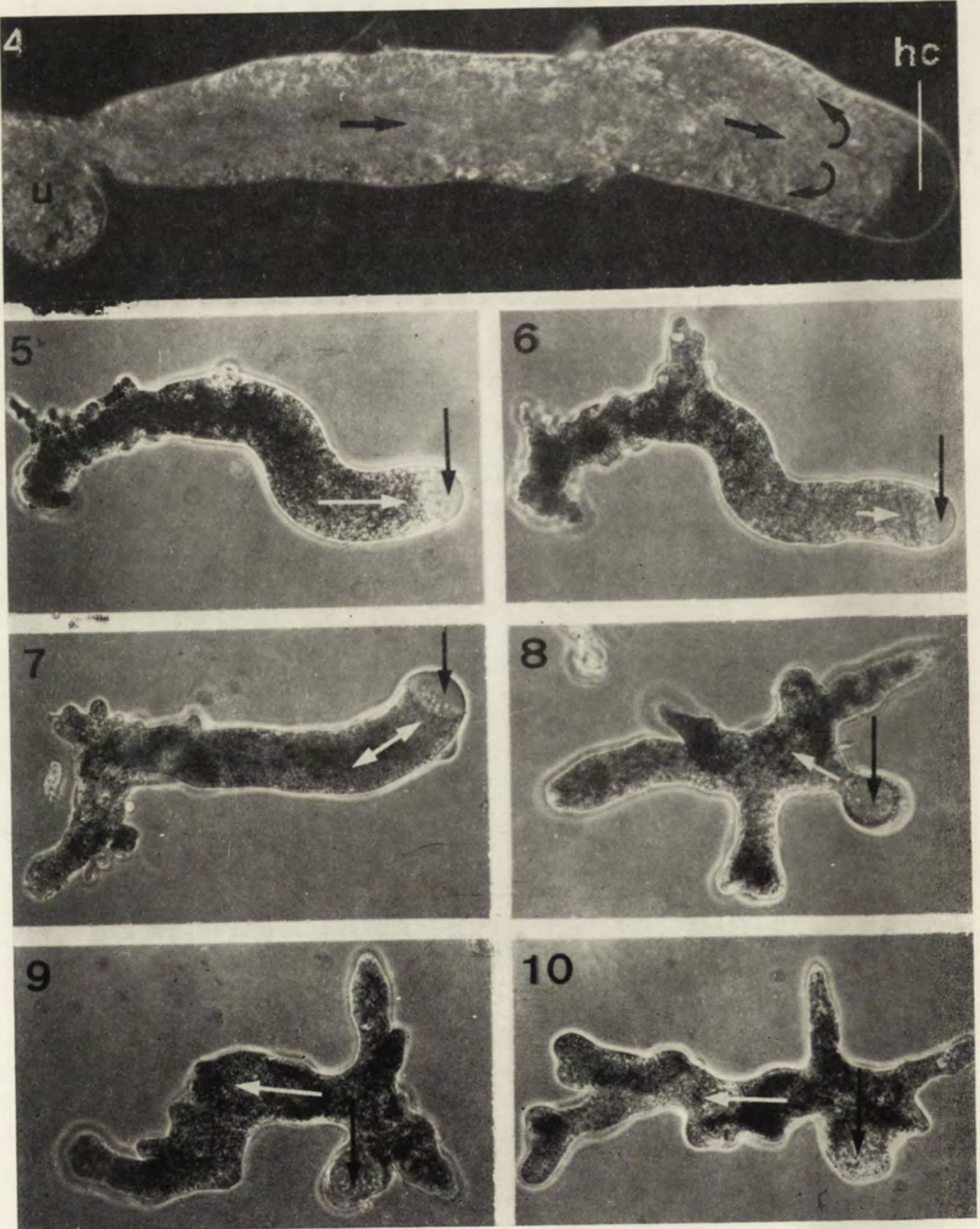

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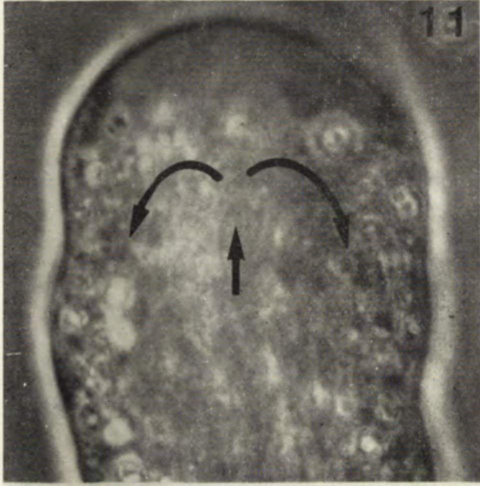

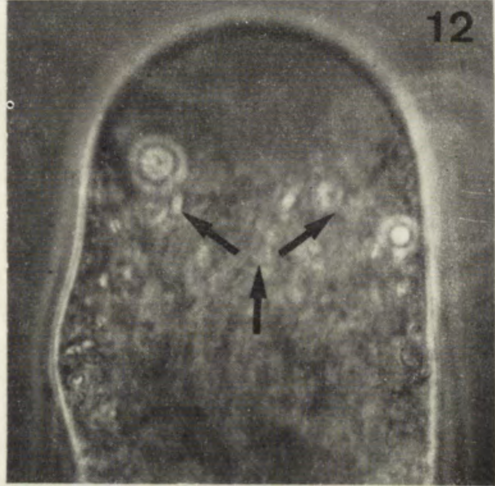

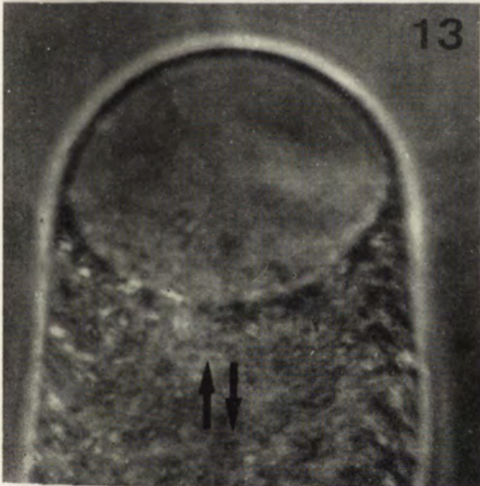

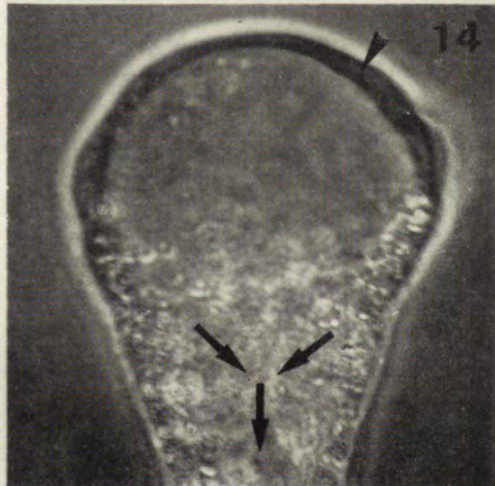

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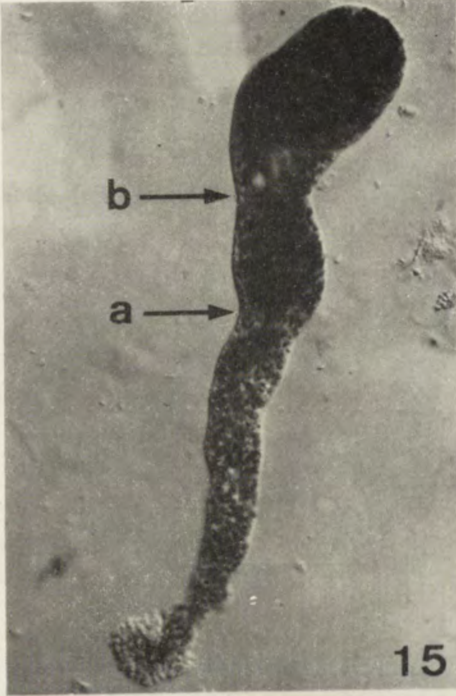

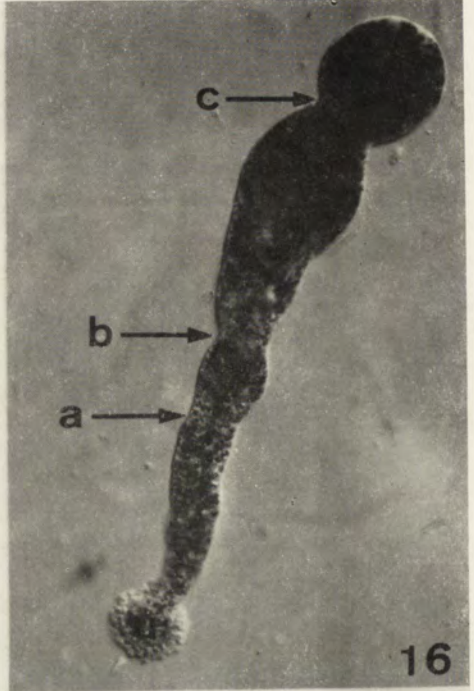

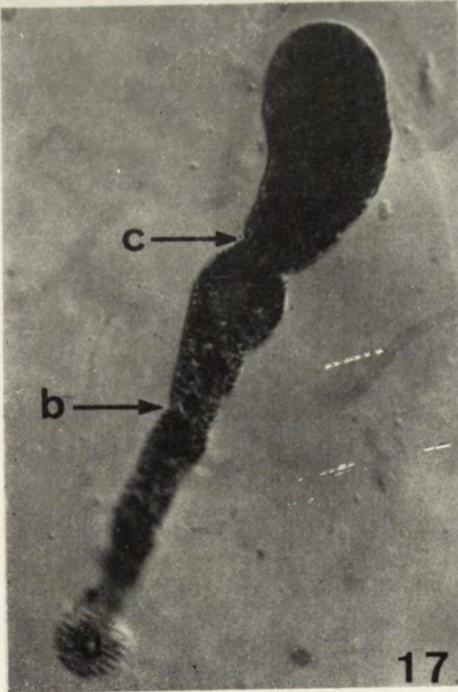

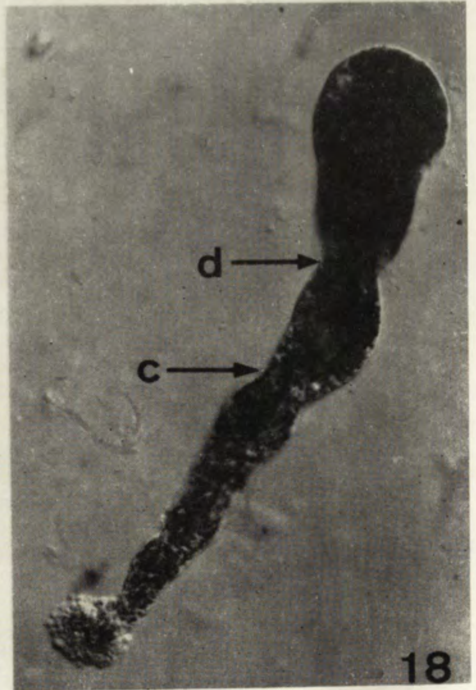

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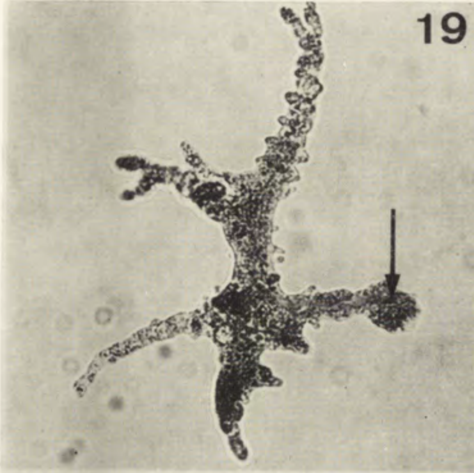

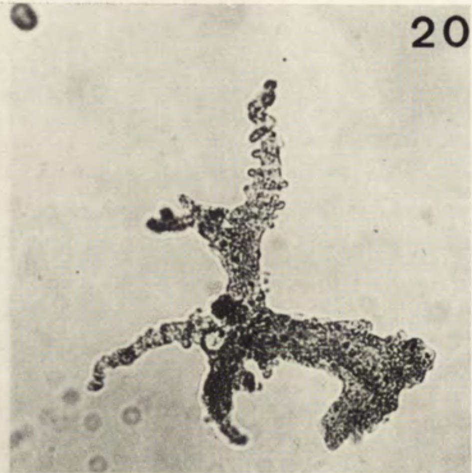

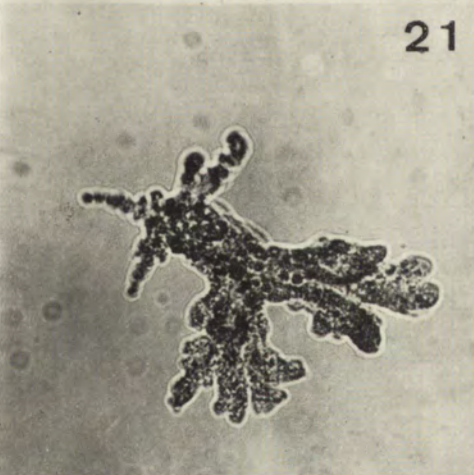

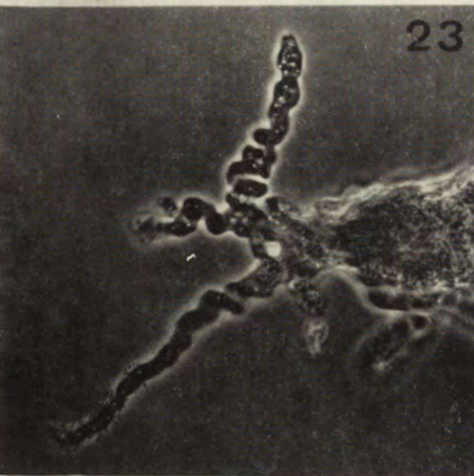

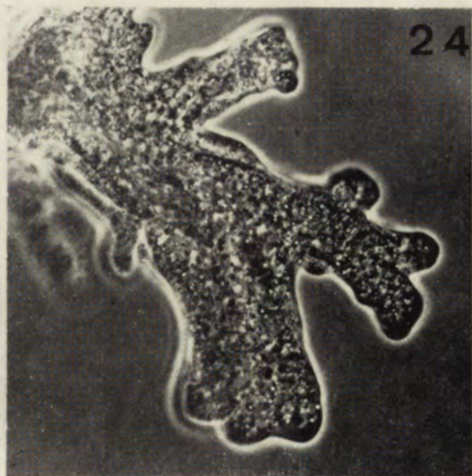

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Reversible Disorganization of Motile Activities in *Amoeba proteus* Induced by General Anaesthetics

Synopsis. Responses of *Amoeba proteus* to ethanol, benzene, and ethyl ether were studied by means of cinematographic techniques. The amoebae reacted differently to ethyl ether than to ethanol and benzene. In a 2% ethanol solution they stopped locomotion, but changes in the cell shape and currents within the granuloplasm continued. Since these two types of cellular motile activities occurred independently of each other, it was concluded that for normal amoeboid locomotion the organized integration of various motile processes in amoebae is necessary. In the ether ethyl — narcotized amoebae a differentation of the granuloplasm into the ectoplasmic cylinder and the endoplasm disappeared, which confirms the earlier conclusions of Brinkley and Daugherty that this anaesthetic also causes liquefaction of the ectoplasm.

While studying the peripheral hyaline zones in the cytoplasm of *Amoeba proteus* the observation was made that cells treated with diluted ethanol solutions show great but reversible disorganization of their motile activities (Korohoda and Stockem 1973, 1975). Recently it has also been demonstrated that the pattern of locomotion and the cell shape in *Amoeba proteus* are correlated with electrical properties of the cell membrane (Braatz-Schade et al. 1973) and that general anaesthetics if applied locally to the amoeba induce positive chemotactic responses (Korohoda 1972). As a continuation of these studies detailed observations were carried out on reversible disorganization of motile activities in *Amoeba proteus* exposed to media containing general anaesthetics. The results of these studies and their bearing on the understanding of motile processes involved in amoeboid locomotion are presented in this communication.

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Material and Methods

Amoeba proteus of the Princeton strain were cultured as described previously (Haberey and Stockem 1971). Specimens chosen for observations were washed in mineral Chalkley's medium. Observations were made with a Zeiss Photomikroskop equipped with a Nomarski interference contrast apparatus, on amoebae placed in three-chamber-slices. These slices allowed the observer to change medium during the experiment, avoiding any changes in the thickness of the observation chamber (Haberey and Stockem 1971). Responses of the amoebae were recorded by both photography and on 16 mm cine-films (Agfa CK 17, Agfa CT 13, Ilford PAN F and Eastman Plus-X). The cine-films were analysed frame by frame on a Lytax Analyser. Ethanol was dissolved in Chalkle's medium. Benzene and ethyl ether were preincubated in closed vessels to obtain saturation.

Results

Preliminary experiments were carried out to determine the effects of chosen general anaesthetics applied at various concentrations to specimens of *Amoeba proteus*. Benzene and ethanol induced similar responses in amoebae, whereas ethyl ether caused slightly variable reactions. Since the concentration of ethanol was the easiest to vary, the majority of the observations were made with this narcotic.

At ethanol concentrations exceeding 10%, the amoebae produced broad hyaline blisters and broke up within a few minutes. Concentrations ranging from 2.5 to 5% caused detachment from glass, a great increase in the hyaline peripheral ectoplasm, and the amoebae assumed the form of a rosette. All movements in the cytoplasm and changes in cell shape gradually ceased. On replacement of the medium by pure Chalkley's medium the amoebae resumed their normal shape and motility within five minutes.

The most interesting responses of amoebae to ethanol were noticed at concentrations ranging from 1.5 to 2%. Hence this concentration was chosen for further more detailed observations. Normal locomotion immediately impaired (Pl. I 1, 2). The hyaline ectoplasm first increased at various points along the periphery, and then numerous pseudopodia were produced. These pseudopodia were strongly attached to the substratum. They were characterized by a broad hyaline ectoplasm. Since they were extend randomly along all sides, the polarization of structure, cytoplasmic streamings, and endoplasm-ectoplasm reactions characteristic of locomoting amoebae disappeared. Gradually pseudopodia became flatter and the cytoplasm differentiad into three zones: external hyaline, central granular, and intermediate hyaline cytoplasm which contained

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small inclusions (Pl. I 5,6 and III 13, 14). As a result the amoebae flattened on the surface of the underlying glass (Pl. I 3–6). Though they did not locomote they continued motile activity. Changes in the cell shape (Pl. III 13–15) as well as endoplasmic currents were still observable. After the replacement of ethanol solution by pure Chalkley's medium (Pl. II 7, 8) the amoebae withdrew their broad hyaline pseudopodia and rounded up. Then gradually numerous short pseudopodia were produced of which one to a few gradually became dominant (Pl. II 9–11). Approximately two minutes after exchange of the medium the amoebae exhibited normal locomotion, functional polarization of structure and cytoplasmic streaming typical of *Amoeba proteus* (Pl. II 12).

The responses to ethanol were followed in detail by cine-film analysis technique. Special attention was paid to analysing the disappearance of polarization and the disruption of normal motile activities in the flattened, narcotized amoebae. The analysis showed that on contact with ethanol, the amoebae at first increased local hyaline ectoplasm at various points along their surface. This was followed by a break in the structure of the ectoplasmic cylinder. At the beginning ectoplasmic granules exhibited random dislocations and then suddenly started to stream into extending hyaline pseudopodia. The extension of pseudopodia was also proceeded by similar intermediate steps. The extension of the hyaline frontal part of the pseudopodia was followed by an influx of granuloplasm. These processes resembled the extension of pseudopodia in some species of small amoebae (Hülsmann 1974). The flattening of amoebae on the glass was caused by random production of numerous pseudopodia, their attachment to glass, and the abnormal influx of granuloplasm into these pseudopodia. In such amoebae, differentiation of granuloplasm into the ectoplasmic cylinder and endoplasm disappeared.

The attached flattened amoebae continued motile activities. Changes in the cell shape and irregular cytoplasmic currents within the granuloplasm were observed. These two phenomena usually took place independently of each other. Changes along the cell periphery concerned only the cortical hyaloplasm and, eventually, the underlying zone of hyaloplasm which contained the small inclusions (Fig. 1). Currents in this zone often did not show any correlation with changes in the shape of the protrusions of the cell periphery.

The granuloplasm of the flattened amoebae possessed quiescent zones separating numerous small streamlets. This region of narcotized amoeba resembled a centrifuged *Chaos chaos* (Heilbrunn 1958) or amoebae treated with ribonuclease (Brachet 1956). Streamlets of granuloplasm, separated by layers of the stationary cytoplasm a few micron thick often ran in opposite directions (Fig. 2). These streamings did not usually show

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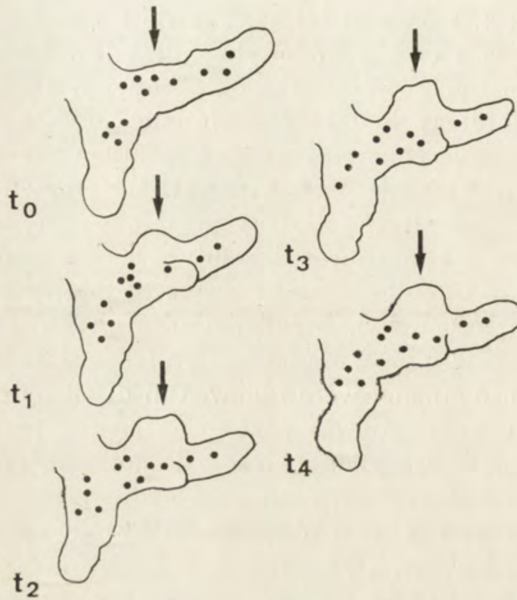

Fig. 1. Morphological changes (arrows) of a hyaline protrusion in a narcotized *Amoeba proteus* without significant streaming processes in the granuloplasm. The dots represent different cytoplasmic inclusions $t_0 - t_4 = 8$ s

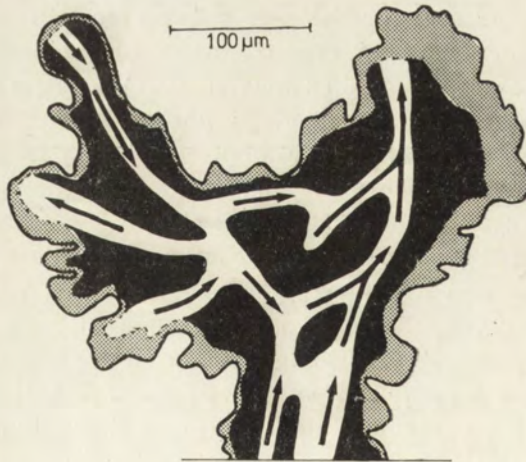

Fig. 2. Network of cytoplasmic streamlets in an ethanol-narcotized *Amoeba proteus*. Raster: hyaline ectoplasm; black: stationary granuloplasm; white: streaming granuloplasm (arrows indicate the streaming direction)

any correlation with changes in the cell contour. If, however, a larger cylindrical protrusion of the hyaline layer of a pseudopodium appeared, the granuloplasm did invade it and streamed to it. Spontaneous reversions of the direction took place sporadically in the streaming granulo-

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plasm. They were sometimes accompanied by a transient jerking motion within the streaming granuloplasm itself. The separation of streamlets and stationary regions of granuloplasm was not permanent and the pathways of certain streams often changed.

As already mentioned, the amoebae exposed to ethyl ether showed different responses. They continued to move with very broad and flattened pseudopodia the number of which decreased with time of exposure. Finally the differentiation of granuloplasm into the ectoplasmic cylinder and streaming endoplasm disappeared completely (Pl. IV). The whole granuloplasm seemed to be disorganized and particles in the granuloplasm usually flowed in one direction, as the amoebae moved monopodially. On the cell periphery a gel-like sheet was clearly visible that the granuloplasm separated from a thin layer of hyaloplasm. Only very small cytoplasmic inclusions passed through this sheet into the hyaloplasm. At higher ethyl ether concentrations the amoebae rounded up and eventually broke. The effects of lower concentrations were reversible upon replacement with pure Chalkley's solution.

Discussion

Observations reported in this communication show that general anaestetics induce great changes in the motile behaviour of amoebae. These compounds act primarily on cell membranes (Berwick 1951, Goldacre 1952, Johnson and Bangham 1969, Seeman et al. 1969). Hence the responses of amoebae may be interpreted as additional evidence for an important role of cell membranes in controlling cellular motile activities (cf. Komnick et al. 1973, Braatz-Schade et al. 1973). However, gradual and segmental disorganization of motile activities in narcotized amoebae suggests that amoeboid movement requires organized integration of a variety of subcellular activities. The behaviour of narcotized amoebae during initial disorganization of their locomotion and after flattening on glass indicates that the changes in the cell contour which occur may be primarily a result of changes in the physical state and activities of the cell membrane and the electron dense layer of hyaloplasm subjacent to it (cf. Korohoda and Stockem 1973, 1975). The initiation of pseudopodium formation also appears to stem from phenomena which occur in the plasmalemma and hyaline ectoplasm and do not appear to be directly related to the streaming of the granuloplasm. In slightly narcotized amoebae, an influx of granuloplasm into extending pseudopodia always follows the events which take place in the outermost layers of the cell periphery. This corresponds to

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earlier observations of Seravin (1967), who found that the resumption of motile activities by thermally shocked amoebae takes place gradually, and changes in the cell contour precede the production of currents in the granuloplasm. Likewise Holtfreter (1948) showed that hyaline fragments of amphibian embryo cells continue locomotion as a result of activities of the cell membrane. The observations carried out on narcotized amoebae, in particular with ethyl ether, appear to confirm earlier studies of Brinley (1928) and Daugherty (1937), who demonstrated that lipid soluble anaesthetics cause a decrease in the viscosity of amoebic cytoplasm. In the ethyl ether-narcotized amoebae, streamings of granuloplasm occur as if they resulted from a differential tension exerted on the endoplasm by the peripheral ectoplasm. In the amoebae narcotized with ethanol, however, many of the currents within the granuloplasm seemed to occur independent of changes in the cell shape. Therefore, the forces originating at the cell periphery are probably not solely responsible for cytoplasmic streaming. The forces which produce multistriated streamings instead appeared to originate in the granuloplasma itself upon its differentiation into streaming and stationary regions. Nevertheless, it is impossible at present to decide whether the observed multistriated streamings are brought about by traction mechanisms, as suggested by Allen for endoplasmic streaming in locomoting amoebae (Allen 1961, 1973), or by hydrostatic pressure mechanisms due to contraction of stationary, presumably more gelled regions of granuloplasm (Heilbrunn 1958). In centrifuged amoebae Heilbrunn has postulated that the streams may be surrounded by their own "cortex" of gelled cytoplasm. This observation corresponds to findings on other amoeba species (Abé 1962, Haberey und Hülsmann 1973).

The present study as well as observations reported earlier (Reinold and Stockem 1972, Braatz-Schade et al. 1973, Stockem 1972, Korohoda 1972, Korohoda and Stockem 1973, 1975) have demonstrated that a locomoting amoeba represents a system in which a variety of subcellular structures contribute to locomotive activities and only their organized integration can bring about cell locomotion. Under experimental conditions such integration may be disturbed and various motile activities may be separated from one another. Significant factors already shown to be involved in the control of functional organization of locomoting amoebae are the functions of the cell membrane and its glycocalyx (Komnick et al. 1973, Braatz-Schade et al. 1973, Korohoda 1972), calcium pumping vacuoles (Reinold and Stockem 1972) and nuclei (Willis 1916, Lorch and Danielli 1950, Jeon 1968). Therefore the phenomenon of amoeboid

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locomotion should not be reduced solely to molecular mechanisms of mechanochemical processes in actomyosin-like proteins or only to the mobility and contractility of the groundplasm.

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ZUSAMMENFASSUNG

Die Reaktion von *Amoeba proteus* auf die Behandlung mit Äthanol, Benzol und Äthyläther wurde kinematographisch untersucht. Der Einfluß von Äthyläther einerseits und Äthanol sowie Benzol andererseits erwies sich dabei als unterschiedlich. So stellten die Zellen in den Benzol- und Äthanol-Lösungen zwar die Fortbewegung ein, zeigten aber weiterhin normale Gestaltsveränderungen und aktive Protoplasmaströmung. Aus der Tatsache, daß beide Phänomene unabhänging von einander auftraten, wurde der Schluß gezogen, daß eine normale amöboide Bewegung nur dann zustande kommt, wenn alle hierfür verantwortlichen Teilprozesse sinnvoll koordiniert sind.

In den mit Äthyläther behandelten Zellen verschwand die sonst vorhandene Organisation des Cytoplasmas in ein hyalines Ektoplasma und ein granuläres Endoplasma. Diese Tatsache bestätigt die Vermutung anderer Autoren, nach denen dieses Anästhetikum die Verflüssigung des Ektoplasmas bewirken soll.

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EXPLANATION OF PLATES I–IV

Plate I (1–6): Disorganization and flattening of *Amoeba proteus* after treatment with 2% ethanol: 1 — specimen showing normal locomotion, 2 — the same amoeba 30 s after incubation in the ethanol solution. The following pictures demonstrate the gradually spreading on glass (3–6). Pictures taken at 60 s intervals. Magnification 150 ×

Plate II (7–12): Reorganization of normal locomotion after removal of ethanol. 7, 8 — amoeba in 2% ethanol, 9–12 subsequent stages in reorganization of normal locomotion after changing the ethanol solution against Chalkley's medium. Pictures taken at 60 s intervals. Magnification: 150 ×

Plate III (13–15): Higher magnifications (1100 ×) of the peripheral regions in a narcotized *Amoeba proteus*. The sequence of pictures demonstrates morphological changes in cell shape within 20 min which are characterized by an increase in the degree of the cell surface folding.

Plate IV (16): *Amoeba proteus* narcotized with ethyl ether. Note the lack of differentation of granuloplasm into the ectoplasmic cylinder and granular endoplasm. (Single frame magnification from a 16 mm cine film). Magnification ca. 500 ×

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Effects of Relaxation and Contraction Stimulating Solutions on the Structure of Cytoplasmic Fibrils in Plasmodia of *Physarum polycephalum*

Synopsis. By application of the solutions of benzamide or ruthenium red and polylysine onto the protoplasmic drops of plasmodia *Physarum polycephalum* it was possible to induce their relaxation and contraction respectively. The effects of these processes on structure of cytoplasmic fibrils were followed with electron microscopy. It was found that both relaxation and strong contraction cause destruction of the fibrils. On relaxation the filaments are separated and then dispersed in the groundplasm whereas on strong contraction the fibrils cleave into nodes in which the parallel arrangement of filaments disappears. The changes in fibril structure are discussed in relation to the processes of cytogel-cytosol conversions.

In the last decade the presence of contractile proteins has been demonstrated in a variety of motile cells (Ishikawa et al. 1969, Pollard 1973, Tatsumi et al. 1973). In these cells the contractile proteins are distributed throughout the entire cytoplasm. They are not organized into permanent and elaborate structures that would correspond to parallel arranged thin and thick filaments in the sarcomeres of striated muscles. Instead, usually only thin cytoplasmic filaments which represent F-actin can be detected with electron microscopical methods (Weissenfels and Schäfer-Dannel 1970, Wessels et al. 1973, Perdue 1973, Comly 1973). A higher organized form are the cytoplasmic fibrils built of bundles of parallel aggregated actin filaments of 60-80 Å in diameter, in plasmodia of slime moulds (Wohlfarth-Bottermann 1962, 1963, 1964, Nagai and Kamiya 1966, Rhea 1966,

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Komnick et al. 1970, 1973). These fibrils can attain a light microscopic order of magnitude. It has been found that they represent a transient differentiation of the groundplasm and appear in the ectoplasm in the regions of plasmodia in which contraction can be assumed. In the flowing endoplasm and in the regions into which the endoplasm flows they are absent or occur in a very small number. The fine structure of the fibrils and the time sequence of their formation have been extensively studied in the drops of protoplasm formed after puncture of plasmodial threads with a needle (Wohlfarth-Bottermann 1964, Komnick et al. 1973).

Since the fibrils occur only in the gelled ectoplasm and disappear on its transformation into the streaming endoplasm, one can expect that their formation and degradation are associated with ecto-endoplasm or cytogel-cytosol conversion. Goldacre and Lorch (1950) suggested that liquefaction of cytogel is a consequence of strong contraction, whereas other authors postulated that it stems from relaxation (Pollard and Ito 1970). According to theoretical considerations of Landau (1959), both contraction and relaxation can lead to conversion of cytogel into cytosol. The experiments presented below were carried out to examine what are the effects of strong contraction and relaxation of cytogel on the fine structure of cytoplasmic fibrils in the plasmodia of *Physarum polycephalum*.

Material and Methods

The plasmodia of *Physarum polycephalum* cultured according to Camps method (1936) were used for the experiments. Preliminary experiments were carried out on the plasmodia migrating on the surface of 1.5% agar in Petri dishes. For more detailed study drops of plasmodial protoplasm prepared as described previously (Wohlfarth-Bottermann 1962, 1964) were used.

To stimulate relaxation of the protoplasmic drops the solution of 20 mM benzamide in 15 mM phosphate buffer (pH 7.0) was applied onto the drops. Contraction of the drops was induced by application of 5 mg polylysine dissolved in 10 ml Ringer medium and buffered to pH 6.5 with TRIS buffer or 10 mg ruthenium red per 100 ml of the same basic medium. The control observations were carried out on the drops embedded in phosphate buffer or Ringer medium without benzamide, polylysine or ruthenium red.

The 10 min old drops of plasmodial protoplasm were treated with the above mentioned solutions for 1, 3 and 5 min respectively. Then the drops were fixed according to Parducz (1952) with 2% OsO_4 + 0.5% $HgCl_2$ (pH 6.5). The fixed drops were embedded in styrol-methacrylate (Kushida 1961). Ultrathin sections made with an Ultratome III LKB were observed and photographed with the electron microscope Philips EM 200.

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Results

It has been reported that benzamide, an anaesthetic, when applied locally to plasmodial threads in calcium-free media causes their local relaxation as judged from observations of endoplasmic flow, electric activity of the plasmodia (Korohoda et al. 1969), and measurements of forces responsible for the endoplasmic streaming (Layrand et al. 1972). Various species and strains of slime moulds differ, however, in their sensitivity to this drug. *Physarum nudum* reacts to 5 mM solutions of benzamide whereas particular strains of *Physarum polycephalum* respond to its concentrations of 20 to 100 mM (Dr Carlile, personal communication). The strain of *Physarum polycephalum* cultured in this laboratory shows a rather low sensitivity and reacts with some delay only to benzamide solutions at a concentration above 50 mM. We expected that the differences in sensitivity to benzamide stem from varying consistency and thickness of the slime coating the plasmodial threads. Hence, solutions with different concentrations of benzamide were applied to the drops of plasmodial protoplasm formed after puncture of the threads with a needle. Such drops regenerated the plasma membrane within a few seconds but did not have so thick a slime coat as did the threads (Wohlfarth-Bottermann and Stockem 1970). It was found that on application of 20 mM benzamide solution to the drops, within two minutes the protoplasm began to flow into them without reversals, like in more sensitive species of slime moulds. Since the formation and fine structure of the cytoplasmic fibrils in such drops had been studied earlier in detail and timed (Wohlfarth-Bottermann 1962, 1963, 1964), they were used for further investigations.

In objects other than slime moulds it was observed that some polycationic compounds depolarizing plasma membrane stimulate contraction of ectoplasm (Ling 1962, Gingell and Palmer 1968, Gingell 1970). In our preliminary experiments it was found that application of polylysine or ruthenium red in calcium containing media to plasmodia of *Physarum polycephalum* induced rapid outflow of the cytoplasm from the treated region, the solution of ruthenium red being more effective of the two. The protoplasmic drops treated with these solutions reacted by rapid and strong shrinkage. Thus, the preliminary light microscope observations confirmed that by treatment of the isolated drops of plasmodial protoplasm with the solutions of benzamide or ruthenium red and polylysine one can stimulate their relaxation or contraction respectively. This offered an opportunity to follow the effects of these processes on the fine structure of cytoplasmic fibrils.

In untreated drops, the fibrils appear in the cortical ectoplasm 3–5 min

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after the drop formation and later persist, increasing in size and number (cf. Wohlfarth-Bottermann 1964). The morphology of the fibrils in the 10 min old control drops treated for 5 min with 15 mM phosphate buffer before fixation is shown in Pl. I A. Identical morphology was exhibited by the fibrils in the drops treated with Ringer medium, i.e., the basic solution used to dissolve in it active compounds had no visible effect on the structure of the fibrils in the cytoplasm of *Physarum polycephalum.*

When 10 min old drops which had a great number of fibrils were treated before fixation for 5 min with 20 mM benzamide, their cytoplasm was almost entirely free of fibrils. Only sporadically some remnants of the fibrils could be found in the cytoplasm. Though their filaments seemed to retain their normal length the remnants of fibrils were much thinner. They were built of few loosely aggregated filaments (Pl. I D). Superficial layers of the drop ectoplasm showed a loose structure and the plasma membrane was smooth (Pl. III A). To follow in more detail the degradation of fibrils the 10 min old drops were fixed after 1 and 3 min of benzamide action. Plates I B and C show that the progressive fibril degradation occurred by longitudinal separation of actomyosin filaments followed by their dispersion in the groundplasm.

The 10 min old drops treated for 3 min with shrinkage inducing solutions of ruthenium red and benzamide showed also great changes in the structure of their cytoplasm. The cortical cytoplasm had a very dense structure. In the drops treated with polylysine (Pl. III C) the plasma membrane was very corrugated, whereas in those treated with ruthenium red (Pl. III B) the plasma membrane produced numerous blebs and protrusions, which suggest its partial destabilization. Through the material which originally built the cytoplasmic fibrils could be easily identified, the structure of the fibrils was strongly altered. The parallel arrangement of the filaments completely disappeared. The processes of fibril transformation could be observed in the drops fixed 1 and 3 min of treatment with the drugs. The fibrils seemed to be broken and the material which built the filaments was concentrated in nodes (Pl. II A–C). In these remnants of the fibrils the parralel arrangement of the filaments had been abolished.

In order to examine whether the shrinkage of the drops induced by ruthenium red can stimulate formation of the fibrils, 0 and 1 min old, i.e., fibril-free drops were treated. Though a rapid shrinkage of these drops could be observed with light-microscope, the electron microscope showed a condensation of the groundplasm, but fibrils were absent. The same was observed when 10 min old drops were treated before fixation for 5 min with benzamide and then for 3 min with ruthenium red.

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Discussion

It has been demonstrated in the previous experiments made in this laboratory that there exists a correlation between the localization of contraction in the ectoplasmic gel of slime mould plasmodia and the occurrence of fibrils built of actomyosin filaments (cf. for review Komnick et al. 1973). However, recently D'Haese and Komnick (1972) have shown that parallel arrangement of actomyosin filaments is not necessary for contraction of thread-models of contractile proteins isolated from rabbit striated muscle. Parallel alignment of filaments only assures a higher speed of contraction and determines its direction. Therefore, the presence of the cytoplasmic fibrils cannot be considered as the only evidence for localization and the only way of cytoplasmic contraction. This was confirmed by the observation that contraction of protoplasmic drops without fibrils (0'age) induced by ruthenium red and polylysine did not induce formation of fibrils but only condensation of the groundplasm.

The results presented show that the fibril degradation may occur during strong contraction as well as during relaxation of the ectoplasm. During relaxation the fibrils disappear due to parallel separation of actomyosin filaments followed by their dispersion in the groundplasm. On the other hand, during contraction the components of the fibril material do not disperse but the fibrils cleave into nodes in which the parallel arrangement of the filaments is destroyed. The same phenomena could be observed during the contraction of glycerinated protoplasmic drops (Achterath 1969). If one accepts that the fibril destruction represents some step in liquefaction of cytoplasmic gel, then the results may be interpreted as confirming the conclusions of Landau (1959). He suggested that the structure of cytoplasmic gel can be destroyed on its strong contraction (cf. also Goldacre and Lorch 1950) but also on its relaxation. However, the interpretation, that the dispersion of the fibrils and the loss of parallel arrangement of F-actin filaments during strong contraction represents a transformation characteristic for isotonically contracted fibrils seems to be more probable. In combined tensiometrical and morphological investigations, Fleischer and Wohlfarth-Bottermann (1975) revealed that isometrically contracted fibrils which were allowed to contract isotonically for only 5 s loose the parallel arrangement of F-actin. The results of these authors are in favour of the thesis, that the isotonically contracted stage of cytoplasmic actomyosin is nonfibrillar. Our results shown in Pl. II can be interpreted on the same way: the fibrillar form of the cytoplasmic actomyosin was lost during a strong, experimentally induced contraction.

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To stimulate contraction we used solutions of compounds whose molecules are strongly positively charged at physiological pH. It has been demonstrated that such compounds depolarize plasma membranes and induce rapid contraction of ectoplasm before they penetrate the membrane (Gingell and Palmer 1968, Gingell 1970). Benzamide can also be supposed to act primarily on the plasma membrane (Korohoda et al. 1969), similarly as other lipid soluble anaesthetics which were shown to induce positive chemotactic responses in amoebae (Korohoda 1972) and degradation of microtubules in *Actinosphaerium nucleofilum* (Allison et al. 1972). Hence, the reported results support the suggestions (Wolpert and Gingel 1968, Braatz-Schade et al. 1973, Korohoda et al. 1970) that changes in the plasma membrane may initiate a chain of events which leads to modification of physicochemical conditions in the cytoplasm, causing in consequence changes in its contractile activity and fine structure.

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ZUSAMMENFASSUNG

Durch Einwirkung von Benzamid- oder von Ruthenium-Rot- und Polylysin-Lösungen auf Protoplasmatropfen der Plasmodien von *Physarum polycephalum* war es möglich, Relaxationszustände bzw. Kontraktionszustände des Cytoplasmas zu erzeugen. Die Auswirkungen dieses experimentellen Einflusses auf die cytoplasmatischen Actomyosinfibrillen wurde elektronenmikroskopisch untersucht. Es zeigte sich, daß sowohl eine starke Erschlaffung als auch eine starke Kontraktion zu einem Abbau der Fibrillen führt. Bei Erschlaffung lockert sich die Parallelanordnung der filamentösen Bausteine auf, die dann im Grundplasma dispergiert werden; bei starker Kontraktion verdichten sich die Fibrillen knotenförmig, wobei ebenfalls die Parallelanordnung der Filamente aufgegeben wird. Die Veränderungen der Fibrillenfeinstruktur werden in Bezug auf das Phänomen der Plasmagel-Plasmasol-Transformation diskutiert.

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EXPLANATION OF PLATES I–III

Pl I: Effects of benzamide on cytoplasmic fibrils in 10 min old drops of protoplasm of *Physarum polycephalum*. Before fixation the drops were treated with: A — 15 mM phosphate buffer, pH 7.0 for 5 min (control), B — 20 mM benzamide in 15 mM phosphate buffer, pH 7.0, for 1 min, C — as "B" but treatment 3 min, D — as above but for 5 min. Note a gradual decrease in the fibril thickness and separation of plasma filaments along the fibril followed by their dispersion in the groundplasm. Parducz fixation, styrol methacrylate embedding, magnification: A — 12 500 ×, B, C, D. — 10 400 ×

Pl. II: Effects of polycations inducing shrinkage of the drops of plasmodial protoplasm on the fine structure of the fibrils. A — disturbance in parallel arrangement of filaments in the fibril of 10 min old drop treated for 1 min with ruthenium red dissolved in Ringer medium (pH 6.5), B — further step in the fibril destruction on treatment for 3 min with ruthenium red, C — disturbance in the fibril structure caused by treatment of the 10 min old drop for 3 min with polylysine. Note an accumulation of fibril components in nodes and destruction in fibril structure. Fixation, embedding as in Pl. I. Magnification: A, B — 10 400 ×, C — 12 500 ×

Pl. III. Effects of relaxation and contraction inducing solutions on the morphology of cortical cytoplasm in the 10 min old drops of plasmodial protoplasm. Prior to fixation the drops were treated with benzamide for 5 min (A), ruthenium red (B), and polylysine (C) for 3 min. Note changes in the density of ectoplasm and in the degree of membrane folding. Fixation and embedding as in Pl. I. Magnification 10 400 ×

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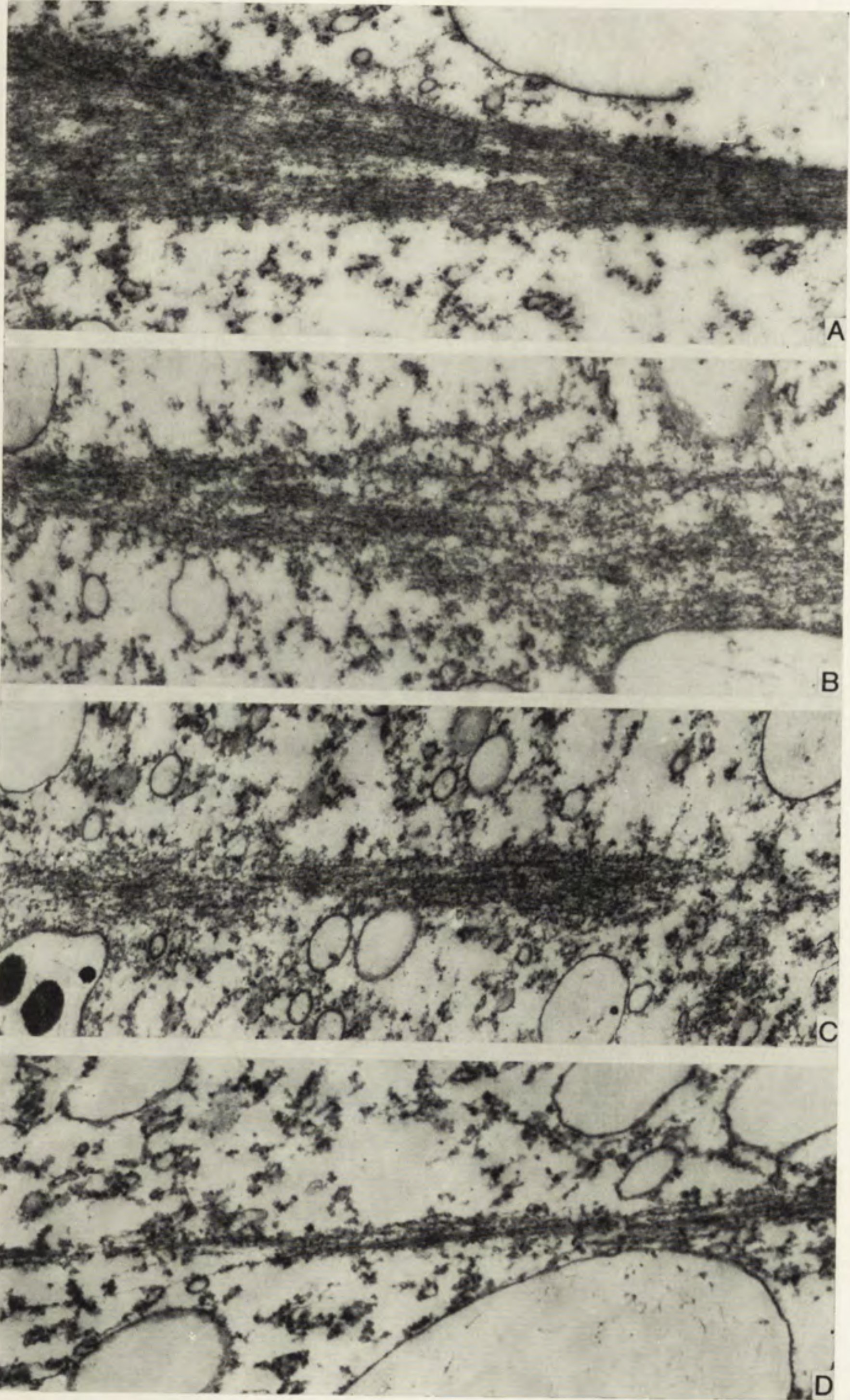

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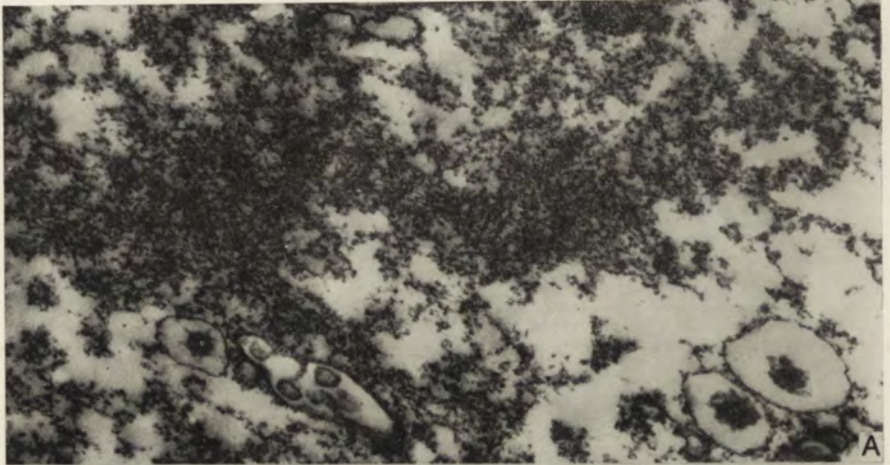

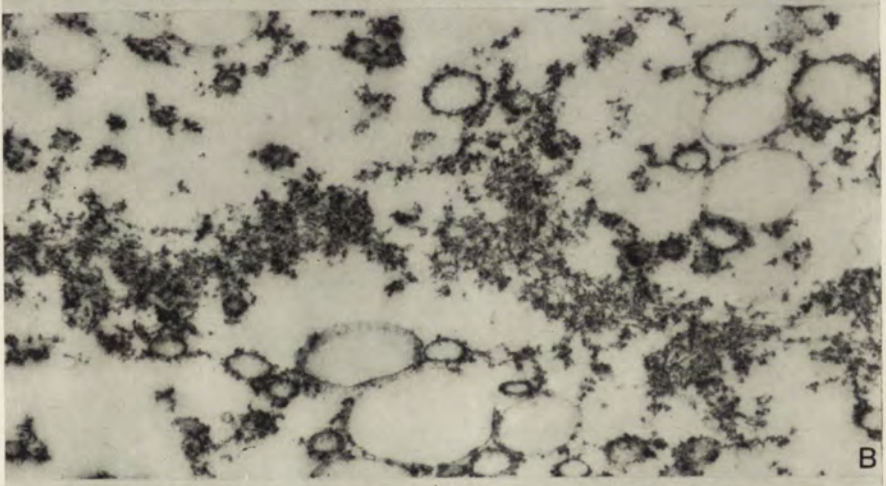

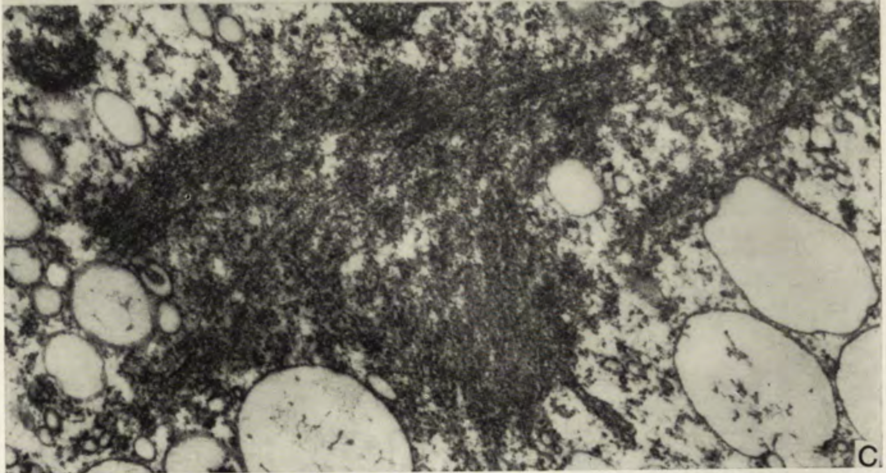

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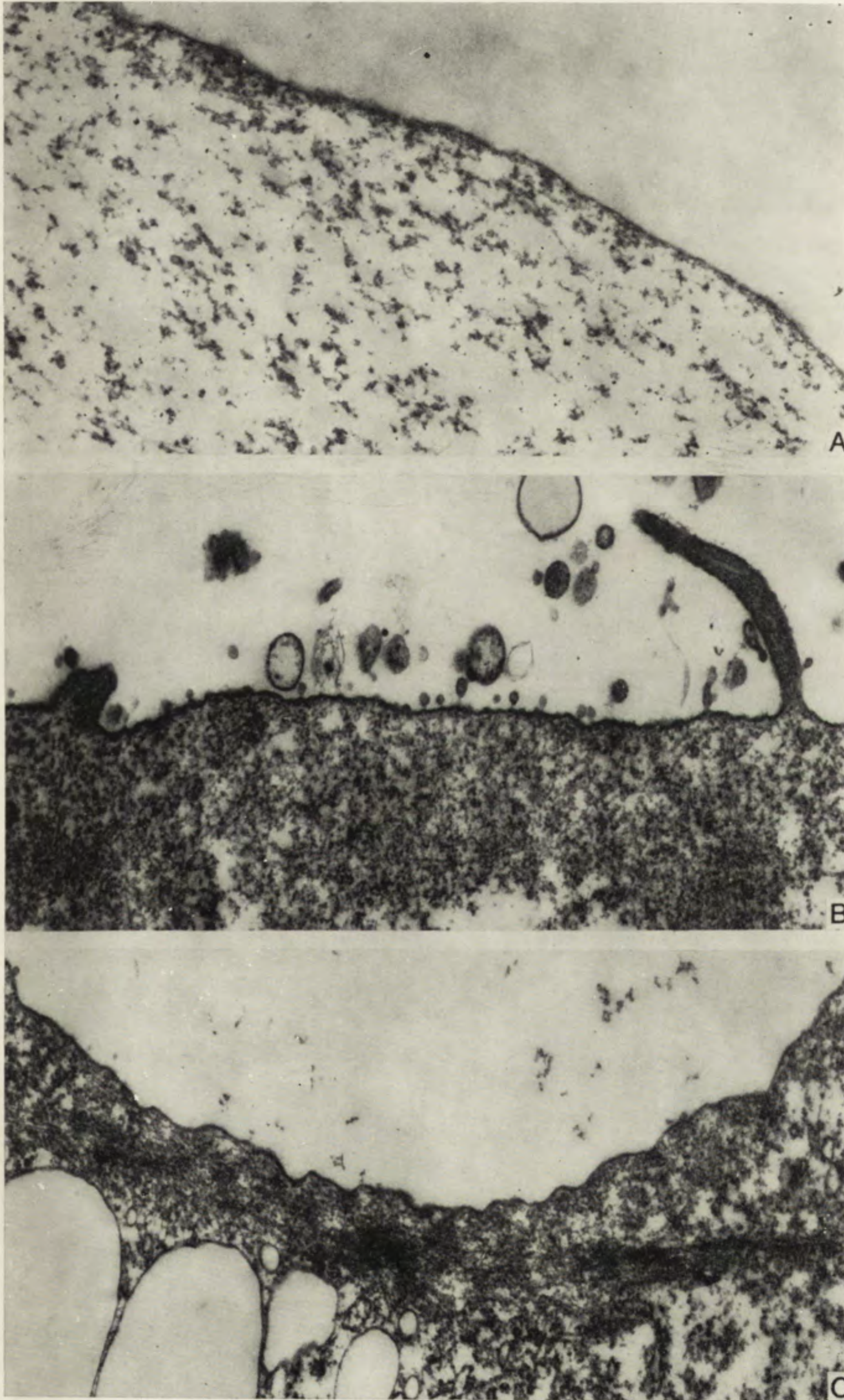

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ACTA PROTOZOOLOGICA

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A Study of Chemotaxis in *Amoeba proteus* I. An Agar Gel Chamber Method for Measuring Quantitatively Chemotaxis in *Amoeba proteus*

Synopsis. A new apparatus by which chemotaxis in *A. proteus* can be quantitatively analyzed is described. This apparatus is made of 2% agar gel and has three separate pools (test pool, amoeba pool and reference pool). The density of amoebae suitable for measuring chemotaxis was about 4 amoebae/mm². Cytochrome c, which can induce foodcup formation, does not show chemotactic activity. The loss of chemotactic activity of an attractant from *Tetrahymena* by trypsin treatment has been also confirmed.

A capillary tube technique has often been used for studying chemotaxis (Pfeffer 1888, Harris 1961). This technique, although invaluable for quantitative analyses of chemotaxis in small organisms such as bacteria (Adler 1973), is not applicable to amoebae because of their large size and slow movement. We have devised an agar gel chamber which permits quantitative studies of chemotaxis in *Amoeba proteus*. This paper describes the experimental conditions suitable for using the agar gel chamber.

Material and Methods

Amoeba cultures: Large number of *Amoeba proteus* were cultured at 20°C in Prescott and James medium (pH 6.0-6.3) containing 6 mg of KCl, 4 mg of $CaHPO_4$, 2 mg of $MgSO_4$ in 1000 ml of distilled water, (method of Griffin 1960) and fed on *Tetrahymena pyriformis*. Amoebae were starved for two days and washed at least three times before use to eliminate contaminants.

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Construction of the agar gel chamber and measurement of chemotaxis: Initially, the agar gel chamber was prepared from Difco Bacto-Agar, which was washed with deionized distilled water according to the method of Konijn and Raper (1961). Due to the lengthy washings required by this technique, we subsequently used Difco Special Agar-Noble. As will be shown, no appreciable differences in the time course of chemotaxis have been found between these two agars.

The agar gel chamber was made by pouring molten agar [usually 2% (w/v) in Prescott and James medium] into a frame made of polymethacrylate resin. The agar chamber consists of three pools partitioned by two thin walls (Fig. 1). A so-

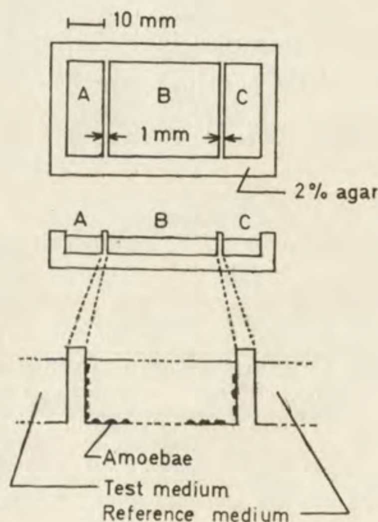

Fig. 1. Agar gel chamber for measuring chemotaxis. A is the pool for a test medium and C, for a reference medium. B is the pool for amoebae

lution (0.8 ml) containing a test substance was pipetted into one of the two side pools (subsequently referred to as the test pool) and a reference solution (0.8 ml) into the other side pool (reference pool). An amoeba suspension (3 ml) was transferred into the middle pool. At ten minute intervals following the initiation of crawling movements on the bottom of the middle pool, the number of amoebae on the wall of the middle pool adjacent to the test pool, and on the other wall adjacent to the reference pool were counted at room temperature. A low-magnification microscope (× 6.3) was used for counting cells on the walls.

Other methods: As test substances, we used the proteinic attractant extracted from *Tetrahymena pyriformis* (Nohmi and Tawada 1974) (this protein is referred to as T-protein), cytochrome c purified from horse heart (purchased from Boehringer Mannheim, Mannheim and Sigma Chemical Co., St. Louis, Mo), trypsinized T-protein, each of which was dissolved in Prescott and James medium, and as a reference, Prescott and James medium was used.

Trypsinization of T-protein was carried out as described earlier (Nohmi and Tawada 1974).

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Results

An example of chemotaxis in *A. proteus* measured by the agar gel chamber is shown in Fig. 2. For this experiment, the chamber was made of 2% agar (washed Difco Bacto-Agar) and T-protein was used as the

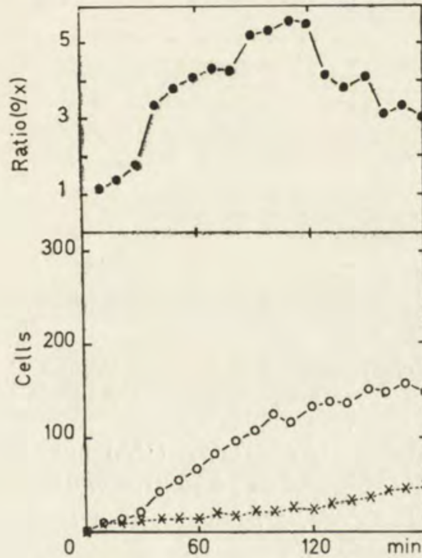

Fig. 2. Time course of chemotaxis. Fig. 2 is the time course in a 2% agar gel chamber. The number of amoebae on the wall of pool B, adjacent to pool A is indicated by the symbol (○) and the number of amoebae on the wall adjacent to pool C indicated by the symbol (x) and these were plotted on the ordinate as a function of time. The ratio, indicated by the symbol (●), was calculated by dividing the former number by the latter, and is plotted on the ordinate as a function of time. The test pool contained T-protein

attractant. The number of amoebae on the wall adjacent to the test pool increased more rapidly than that on the other side wall, furthermore, in the former, the number leveled off after 180 min while the latter continued to increase. The ratio of amoebae on the wall adjacent to the test pool to those adjacent to the reference pool reached a maximum at 120 min, and then decreased. Similar results were obtained when Difco Special Agar-Noble was used instead of the washed agar.

When both the test pool and the reference pool contained Prescott and James medium, the number of amoebae on the two side walls was the same, and the ratio of the cells was always unity (Fig. 3).

Effect of agar concentration on chemotaxis: A or 3% agar chamber produced the same time course of chemotaxis as obtained

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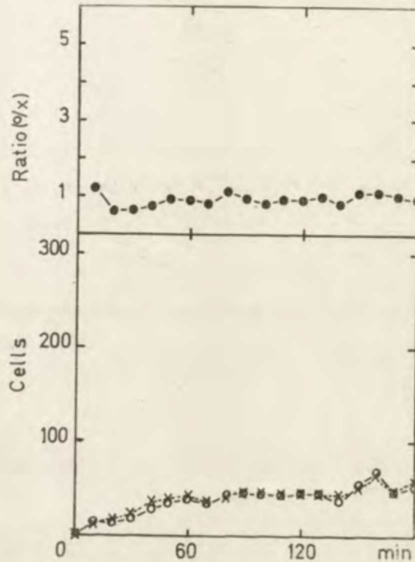

Fig. 3. Time course of chemotaxis when pools A nad C contained Prescott and James medium

for the 2% agar chamber. However, a (1%) agar chamber was too soft to support the weight of amoebae, and the walls were not rigid enough to remain vertical. Therefore, 2% agar Difco Special Agar-Nogle chambers were used in the following experiments.

D e p e n d e n c e o f t h e t i m e c o u r s e o f c h e m a t a x i s o n a m o e b a c e l l d e n s i t y: Chemotaxis was measured at various densities of amoebae in the middle pool of the agar chamber. The results are shown in Fig. 4a for 1.6 amoebae/mm^2, Fig. 4b for 3.6 amoebae/mm^2, and Fig. 4c for 7.4 amoebae/mm^2. The maximum ratio of amoebae on the two side walls was density dependent. The smaller the density, the greater the ratio. At a density of less than 1.6 amoebae/mm^2 the number of amoebae on the walls was small and consequently statistical errors in the calculated ratios were large. At densities greater more than 7.4 amoebae/mm^2 the ratio was not greater than 2 within 3h. Thus a density of about 4 amoebae/mm^2 was most suitable for experimental study.

E f f e c t o f t h e c o n c e n t r a t i o n o f a t t r a c t a n t o n c h e m o t a x i s: Chemotaxis was measured two different concentrations of T-protein: 0.06 and 0.02 mg/ml (estimated from the standard curve for bovine serum albumin). In each case (Figs. 5a and b), the number of amoebae that migrated toward the test pool was greater than the number of amoebae that migrated toward the reference pool within 1 h after the beginning of the experiment. When the concentration of attractant was

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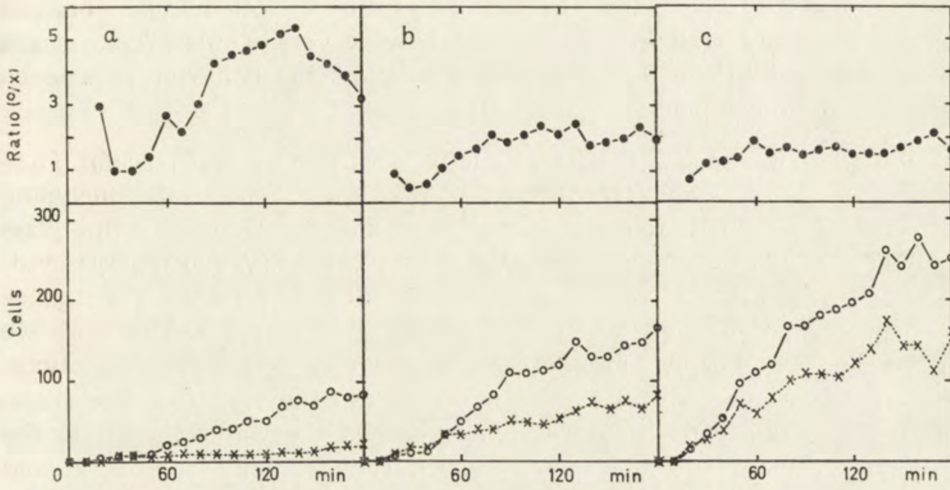

Fig. 4. Dependence of the time course of chemotaxis on the density of amoebae. Time course of chemotaxis at a cell density of 1.6 amoebae/mm² is shown in Fig. 4 a, at 3.6 amoebae/mm² in Fig. 4 b and at 7.4 amoebae/mm² in Fig. 4 c

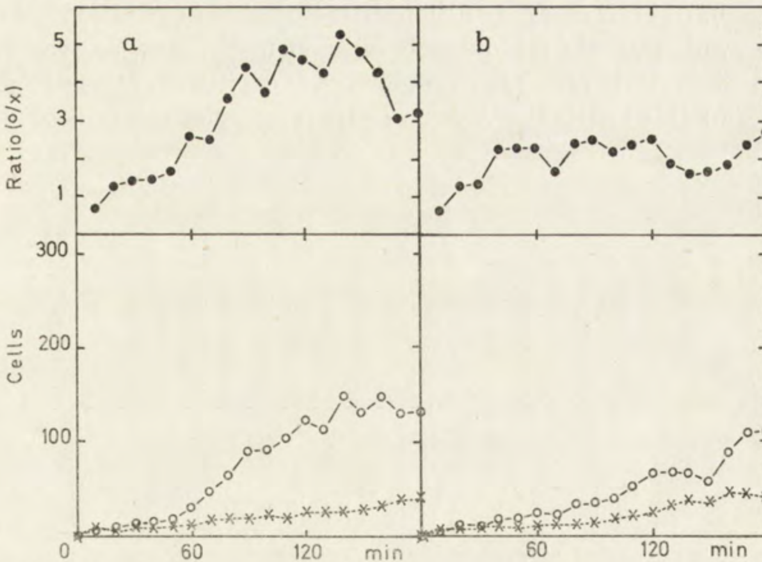

Fig. 5. Effect of attractant concentration on amoeba chemotaxis. Fig. 5 a is the time course of chemotaxis when the concentration of T-protein was 0.06 mg/ml and Fig. 5 b is the time course when the concentration was 0.02 mg/ml

0.06 mg/ml, however, the amoeba moved much faster to the test pool than when the concentration was 0.02 mg/ml. At a protein concentration of 0.02 mg/ml, the ratio of amoebae on the two walls leveled off within 1 h. At a concentration of 0.06 mg/ml, however, the ratio continued to

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increase for 2.5 h. Note that the time courses of the cell number-increase on the reference wall in Figs. 5a and b were very similar. This means that attractant in the test pool does not affect the behavior of amoeba near the reference pool.

Cytochrome *c* as a substance inducing foodcup formation: Seravin (1968) reported that many chemicals, including cytochrome *c* induced foodcup formation of amoeba. He used a fine glass capillary (inside diameter of 10-30 μm) filled with 2% (20 mg/ml) cytochrome *c* to test the effect of the chemical. All the chemicals that induce foodcup formation might not be attractants in *A. proteus*. Therefore we first tested whether cytochrome *c* could be an attractant for chemotaxis. When cytochrome *c* of 10 mg/ml was put into the test pool, the shape of the amoeba became round as it approached the wall adjacent to the test pool. Subsequent movement of such rounded amoebae ceased and the number of amoebae on this wall was smaller than that beside the reference pool. When cytochrome *c* of 1 mg/ml was put into the test pool, amoebae on the wall adjacent to the test pool did not become round but amoebae on this wall were at all times the same as those on the other wall (Fig. 6). In both cases, diffusion of the coloured cytochrome *c* through the thin wall into the middle pool was visible. Likewise, we observed that amoebae did not migrate towards capillaries (inside diameter 200–500 μm) filled with 1.5% agar containing cytochrome *c* of 0.5, 1 or 10 mg/ml.

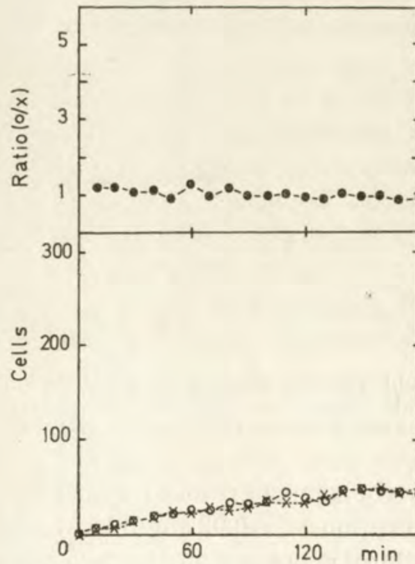

Fig. 6. No chemotactic effect of cytochrome c (1 mg/ml) in the agar gel chamber

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Loss of chemotactic activity of T-protein by trypsinization: Chemotactic activity of T-protein was lost following trypsin treatment (Nohmi and Tawada 1974). This was confirmed also by the agar gel chamber method (Fig. 7).

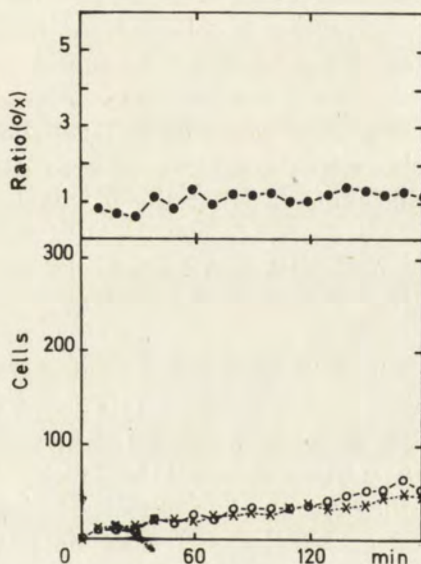

Fig. 7. Loss of chemotactic activity of attractant treated with trypsin

Discussion

In the experiments shown in Fig. 4c, the ratio of cell numbers was greater than 1.5 but did not exceed 2. At lower densities of amoebae, the ratio was greater than 2 as shown in Figs. 4a and b. In the three experiments of Figs. 4a, b and c, the protein concentration of the attractant in pool A was identical and the chemotactic effect of the attractant was apparent. Hence, in general we can safely consider that a test substance has chemotactic activity when the ratio of cells on the wall adjacent to the test pool to those adjacent to the reference pool is greater than 1.5.

The time course of cell-number increase on the walls was not dependent upon the agar concentration (ranging from 1 to 3% of the chamber). This might indicate that the time of diffusion of the attractant through the thin wall from pool A to pool B is hardly a rate-limiting factor on the accumulation of amoebae on the walls.

A cell density of about 4 amoebae/mm² is also suitable for rapid counting with small statistical errors.

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The shape of amoebae became round as they migrated towards a pool containing a concentrated solution of cytochrome *c*. Since amoebae also take round shapes when suspended in a concentrated solution of cytochrome *c*, this means that cytochrome *c* probably diffuses into the middle pool of the chamber. Diffusion of cytochrome *c* into the middle pool was also visible, since cytochrome *c* is coloured. At lower concentrations of cytochrome *c* amoebae did not become round in shape, and did not show chemotactic activity.

Using a fine glass capillary, Seravin (1963) showed that application of concentrated cytochrome *c* to a localized area of an amoeba induced foodcup formation. This indicates that the mechanism of foodcup-induction by cytochrome *c* is different from that involved in chemotaxis. This view is supported by observations (Edwards 1921) that some acids and bases can induce pseudopod formation but do not have chemotactic activity.

Until now capillary tube technique has been the only suitable method for studying chemotaxis in amoebae. In this technique a fine glass capillary, containing agar to delay diffusion of attractant, is usually used to examine whether a substance contained in the capillary can induce foodcup formation on the surface of an amoeba. However, foodcup formation might not be used as a criterion for chemotactic activity of a substance, as pointed from experiments of the chemotactic activity of cytochrome *c*. With a capillary of a larger diameter, chemotactic activity can be tested by counting the number of amoebae that migrate to the tip of capillary (cf., Nohmi and Tawada 1974). However, this is not a quantitative measure because of the small number of migrating amoebae. The agar gel chamber is more suitable for quantitative analyses than the capillary tube technique.

We have used the agar gel chamber to test the changes in chemotactic activity of T-protein when the conformation of the protein is altered. The results are described in the following paper.

ACKNOWLEDGEMENT

We thank Professor H. Shimizu for his encouragement during this work.

RÉSUMÉ

Un dispositif nouveau a été conçu pour procéder à l'analyse quantitative de la chimiotaxie chez *Amoeba proteus*. Il est produit en gel d'agar 2% et il comporte trois chambres séparées (pour la solution examinée, pour les amibes et pour la solution contrôle).

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La densité d'échantillon convenable pour l'analyse de la chimiotaxie est d'environ 4 amibes par mm². Le cytochrome c qui peut stimuler la formation des enfoncements alimentaires, ne provoque pas de réponse chimiotactique. On a confirmé également la perte du pouvoir chimiotactique attractif, d'un extrait de *Tetrahymena* après son traitement par la trypsine.

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A Study of Chemotaxis in *Amoeba proteus* II. Requirement of a Specific Conformation of an Attractant in Amoeba Chemotaxis

Synopsis. The effect of heat-denaturation of a proteinic attractant extracted from *Tetrahymena pyriformis* on chemotactic activity in *Amoeba proteus* has been investigated. The chemotactic activity was measured by the agar gel chamber method described in the preceding paper and conformational changes of the attractant was measured by circular dichroism. The conformational change of attractant closely correlated with the loss of the chemotactic activity. This close correlation suggests that the interaction between the attractant molecule and the amoeba surface membrane is specific.

The initial step of stimulus-response systems of living organisms is the reception of an external stimulus. In chemoreception, there is a close correlation between the molecular structure of a stimulant and its stimulating effectiveness. In the blowfly the stimulating effectiveness of a series of derivatives of D-glucose on the sugar receptor was studied (Evans 1963). It was shown that D-fructose is effective only in the β-D-fructofuranose form. This suggests a specificity of interaction between the sugar and the receptor. It was also shown that amoebae of some *Dictyostelium* species are attracted by adenosine cyclic 3', 5'-monophosphate (cyclic AMP), and to a lesser extent, by the analogues of this nucleotide (Konijn and Jastorff 1973). They concluded that the molecular receptor systems for cyclic AMP of the amoebae are highly sensitive to stereochemical alteration at the 5' position of cyclophosphate ring. This also shows a specificity.

We extracted a protein from *T. pyriformis* (Nohmi and Tawada 1974), which was an attractant for amoeba chemotaxis. The study of the relative chemotactic effectiveness of the partially denatured atrractant is presented in this paper. Our results show that the interaction between the amoeba membrane and the attractant is specific.

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Material and Methods

Amoeba cultures: *Amoeba proteus* was cultured as described in the preceding paper (Nohmi and Tawada 1976).

Preparation of test substances: The method for extracting the chemotactic substance from *T. pyriformis* was described in detail previously (Nohmi and Tawada 1974). Heat-treatment of the attractant was carried out without and with using 8M-Urea as the denaturant. In the former case, the attractant was boiled at 100°C for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h. The seven test substances after the heat-treatment were immediately placed in an ice bath. They were kept at room temperature for 0.5 h before the beginning of chemotactic test and CD measurement. In the latter case, the attractant containing 8M-Urea was incubated at 50°C for 2 h, dialyzed exhaustively and concentrated till the same concentration as untreated attractant was obtained.

Other methods: Amoebae were starved for two days and washed at least three times before use to eliminate contaminants.

The chemotactic activity was tested at room temperature by using the agar gel chamber described in the preceding paper.

CD measurements were carried out with a Jasco ORD/UV-5. The scale setting of 5×10^{-4} dichroic absorbance difference per 1 cm on the chart was used. All recordings were carried out at ambient room temperature of 20°C.

To examine whether a test substance passed through the thin wall of an agar chamber, the following procedure was used. Following each experiment, the chamber was rinsed to remove protein and amoebae from the agar surface. Then the rinsed agar gel chamber was sliced vertically so as to obtain sections of the two thin walls. The slice was dried and passed through 20% aqueous acetic acid containing 0.5% Amido-Schwartz dye. As the agar does not stain, the protein which takes up the stain can be observed.

To examine the net change of charge of the test substance, electrophoresis on cellogel was caried out. Cacodylic acid-imidazole buffer of pH 5.3 and Tris-veronal buffer of pH 7.0 were used. The staining solution was 45% methanol–10% acetic acid containing 0.5% Amido-Schwartz, and the decolorant was 47.5% methanol–5% acetic acid.

Results

Figure 1 shows the CDs of native attractant (i. e., attractant before heat-treatment) (Curve *1*), the attractant boiled at 100°C for 3 h (Curve *2*) and Prescott and James medium which was the solvent of these test substances (Curve *3*) in the form of actual CD tracings. In all seven samples a negative peak was observed at 220-223 nm. The readings of a dichroic absorption difference at 200 nm were plotted against the heat-treatment time (Fig. 2). The ΔO.D. gradually decreased after 30 min boiling. Since the amplitude of the Cotton effect is related to the amount of helix content, it is likely that the longer the attractant is boiled, the less order it

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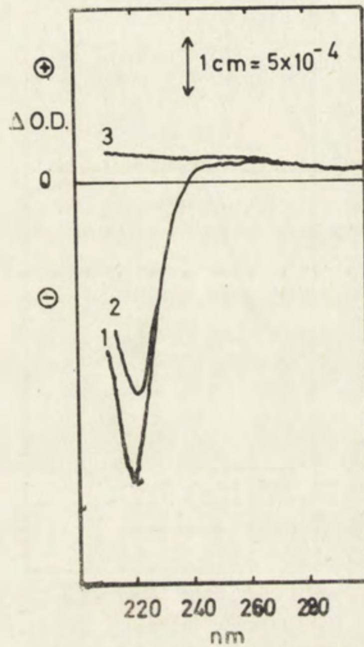

Fig. 1. CDs of untreated attractant (Curve *1*), attractant boiled for 3 h (Curve *2*) and Prescott and James medium (Curve *3*), in the form of the actual CD tracings. (Absorbance at 280 nm was 0.24)

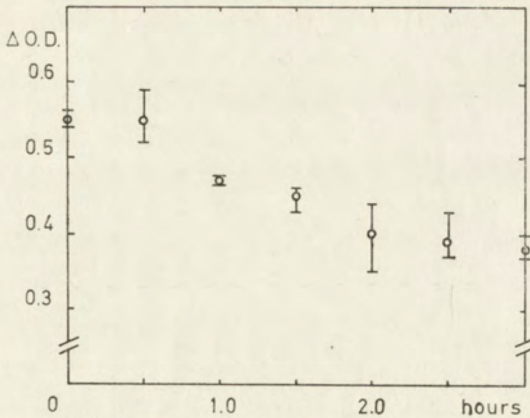

Fig. 2. Δ0.D. of the CD at 220 nm of attractants boiled for various times was plotted on the ordinate against time. These samples had the same concentration (Absorbance at 280 nm was 0.24)

has. Figure 3 shows the CD of 8M-Urea-treated attractant. The structure of attractant after the treatment was also altered.

Figure 4 shows the time course of chemotaxis in the agar gel chamber with heat-treated attractants. Figure 4a shows the time course when

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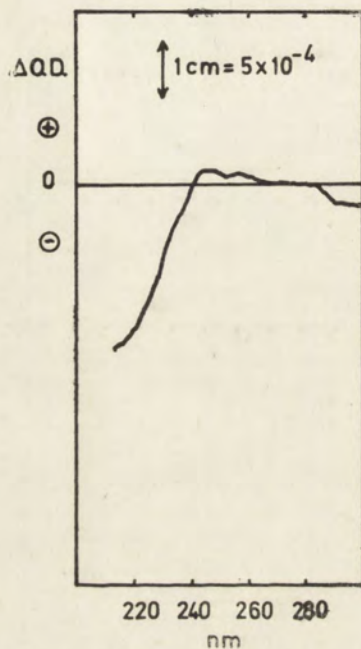

Fig. 3. The CD of attractant treated with 8M-Urea in the form of an actual CD tracing. (Absorbance at 280 nm was 0.06)

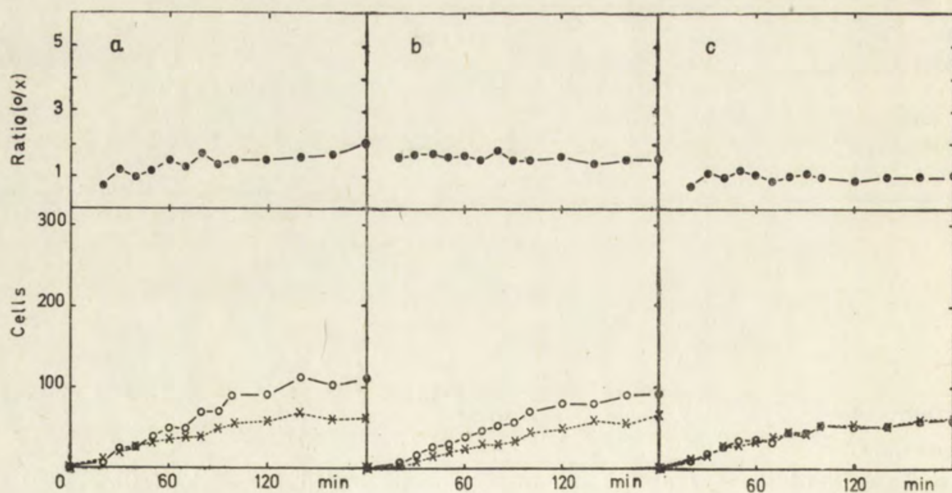

Fig. 4. Time course of chemotaxis (Absorbance at 280 nm was 0.94). Abscissa and ordinate in both upper and lower graphs are same as in "A study of chemotaxis in *Amoeba proteus* I. An agar gel chamber method for measuring quantitatively chemotaxis in *Amoeba proteus* (Nohmi and Tawada 1976)". Amoebae on the wall adjacent to the reference pool (x). Amoebae on the wall adjacent to the test pool (○). Figure 4 a is the time course of chemotaxis with untreated attractant; Fig. 4 b is the time course with the attractant boiled for half an hour; and Fig. 4 c is the time course with the attractant boiled for 2 h

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untreated attractant was used. Figure 4b shows the time course with 0,5 hour-boiled attractant and Fig. 4c shows the time course with 2 hours-boiled attractant. The chemotactic activity became weaker as the heat-treatment time was increased beyond half an hour. The activity was completely lost by heat treatment for 2 h. Figure 5 shows the time course of chemotaxis when Urea-treated attractant was used. This attractant had no activity.

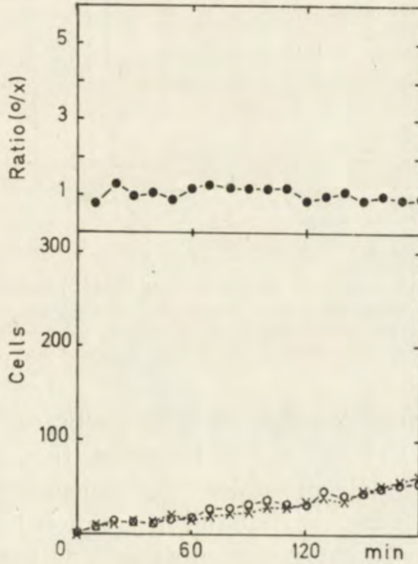

Fig. 5. Time course of chemotaxis with attractant treated with 8M-Urea

Staining of the sliced agar showed that the diffusion of attractant through the thin wall from pool A into pool B occurred. Electrophoresis showed that heat treatment of the attractant did not change the net charge of the attractant (Fig. 6).

Discussion

Our results show close correlation between the loss of chemotactic activity and the conformational change of attractant: the chemotactic activity of attractant was lost when the helix content of the attractant was decreased to some extent.

The net charge of attractant did not change when the conformation was altered by heat treatment. The heat-denatured attractant diffuses out into pool B equally as well as the untreated attractant. Therefore, the

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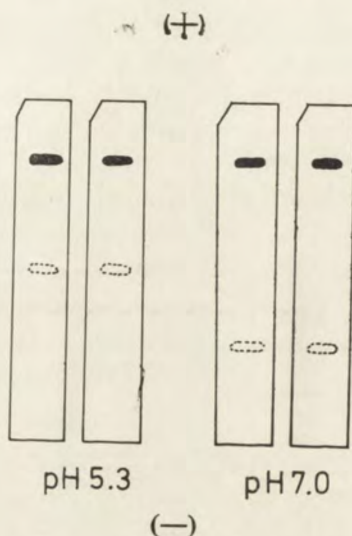

Fig. 6. Electrophoresis on cellogel of untreated and heat-denatured attractants at pH 5.3 and pH 7.0. At both pHs the positions of untreated attractant (right side) and heat-treated attractant (left side) after electrophoresis are indicated by the spots, and the positions at the start are marked by the dashed lines

close correlation between the loss of chemotactic activity and the conformational change of the attractant indicates that an amoeba can not "sense" the attractant molecule unless the molecule has a special (native) tertiary structure. This means that the interaction between the attractant molecule and the amoeba surface membrane is as specific as that between stimulant molecules and chemoreceptors in higher organisms.

ACKNOWLEDGEMENT

We wish to express our thanks to Prof. H. Shimizu for his encouragement.

RÉSUMÉ

On a étudié l'activité chimiotactique chez *Amoeba proteus* stimulée par une protéine attractive extraite de *Tetrahymena pyriformis,* après sa dénaturation par la chaleur. L'intensité de la chimiotaxie était méasurée par la technique des chambres en gel d'agar décrite dans l'étude précédence et les changements de la conformation du facteur attractif était mésurés par la méthode du dichroïsme circulaire. Ces changements sont strictement liés avec la perte de l'activité chimiotactique. Cette corrélation étroite suggère une interaction spécifique entre la molécule du facteur attractif et la surface de la membrane de l'amibe.

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Co-axial Motion of the Semi-rigid Cell Frame in *Amoeba proteus*

Synopsis. The locomotion of polypodial amoebae, well attached and not stimulated, has been recorded by means of the double-exposure and the long-exposure photography, and of the time-lapse cinematography as well in the horizontal plane as in the side-view. The attachment to the substrate by supporting knobs or pseudopodia in the zone, distant by 1/3 of the body length from the front and 2/3 from the rear edge, is most common and most efficient for locomotion. The ectoplasmic tube is not stationary. Under these conditions of cell adhesion, it shortens and is pulled forwards behind the limited attachment zone, whereas it expands and is pushed, also forwards, in front of it. The motion of the ectoplasmic tube is reflected in the granule movements and in changing the position of the external contour of amoeba. The ectoplasm remains stationary only temporarily for a short time in the limited attachment zone, which itself continuously changes its position in respect to the substrate and in respect to amoeba's morphology. The result is a real crawling movement of the whole semi-rigid cell frame, corroborating the locomotion. Specimens occasionally anchored by the uroid or, more commonly, by the tip of a growing frontal pseudopodium cannot effectively lomocote, and there appears a non-effective compensatory backward motion of the whole cell frame, which creates the impression of a fountain streaming.

Introduction

It is well known that the recent theories of the amoeboid movement based on the gel tube contraction (e. g., Goldacre and Lorch 1950, Goldacre 1961 and 1964, Marsland 1964), and the theory of the frontal contraction (Allen 1961 a and b) essentially disagree as to the site of action of the motive force in the cell. According to the first theory, the contraction takes place in the stationary ectoplasm and

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results in pushing the moving endoplasm, and according to the second, the contraction in the moving endoplasm pulls it against the stationary ectoplasm. It becomes therefore obvious that, unexpectedly, both controversial theories are based on the same common fundamental presumption: the endoplasm is the only fraction of the cell body which actually moves during the cell locomotion in amoeba. Advancing of the ectoplasmic tube is not a real movement but only a growing on at the front and a disintegration at the rear. If so, we are forced to conclude that the whole transport of material in the moving cell is effected exclusively through its interior, and as a consequence, to believe that it would be enough to reveal the mechanism of the endoplasmic streaming for understanding the locomotion.

This manner of thinking is basically consistent also with the view of Goldacre (1961) postulating that even the cell membrane is stationary in a moving amoeba, and is transported forwards uniquely in a disintegrated form by the intracellular streaming. However, this hypothesis has been replaced in the past decade, at least for *Amoeba proteus* [1], by a theory stating that the cell membrane actually moves forwards by a folding-unfolding process (Czarska and Grębecki 1966, Haberey, et al. 1969, Stockem et al. 1969).

The aim of this study is to demonstrate that in general the locomotion of amoeba cannot be reduced in its interpretation to the intracellular transportation of material by the cytoplasmic streaming. As a matter of fact, a migrating amoeba performs regularly plenty of other movements which cannot be described in the terms of gradual disintegration and reconstitution of a stationary ectoplasmic tube. For example, the lateral pseudopodia may move with their bases forward or backward in respect to the substrate, or bend in any point, the whole cell may effect short lateral movements perpendicular to the main axis of the endoplasmic stream, many vertical movements, or some turning at the spot. These movements which make the cell change its position and/or its shape independently of the cytoplasmic streaming, are so commonly and easily seen under usual conditions that it is impossible to explain why are they neglected in all theories of amoeboid movement, if not by a common fascination by the ectoplasm-endoplasm cycle in amoeba. To fill this gap an effort should be made to describe these movements, to distinguish between the major types of them, to understand their mechanism, and to integrate their interpretation into the general theory of amoeboid locomotion.

[1] The concept of the stationary condition of the cell membrane is sometimes still supported in the case of the locomotion of tissue cells (Harris 1973).

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For the purpose of convenience, to all this diversity of movements will be given here a common name of autotraction. This term is adopted from Jahn and Bovee (1965), however, in a very different meaning und without any relation to the taxonomical conclusions drawn by these authors. It should simply mean here that the cell or a part of it changes its position as a whole, regardless of the fact that the intracellular transportation of material is simultaneously acting inside it. In other words, and in contrast to the movement of the semi-fluid component of the cell, autotraction is the movement of the semi-rigid frame of the cell body[2].

The autotraction phenomena may be subdivided first into two large categories: the co-axial movements in which the semi-rigid cell structures advance following the same course as the endoplasmic streaming or are retracted in the opposite direction, and the non-axial movements which are effected in any other direction not corresponding to the intracellular flow. The co-axial movements are the subject of the present study.

The autotraction of the semi-rigid cell frame in amoeba has two aspects, one microstructural and another one macrostructural, which can be observed separately with different techniques but in fact are intimately related one to another. At the microstructural level the cell frame displacements are expressed by the movement of granules of the ectoplasmic tube, and at the macrostructural one by changing position of pseudopodia, of the uroid, and of the other small projections of the trunk. Both phenomena were recorded and are analysed in this study for revealing the overall pattern of movements of the cell frame during the locomotion of amoeba.

Material and Methods

The cultures of *Amoeba proteus* were grown in the Chalkley medium with *Colpidium* added as food. The samples taken for recording the movement were washed with the clear fluid from the upper layers of the same culture, i.e., without changing any physico-chemical properties of the medium.

All the amoebae, analysed in this study were well attached to the substrate, manifested different degree of polypodiality, and were not stimulated. In other words, they presented the body shape and type of locomotion most common in

[2] The term of the semi-rigid frame is used here in its physical meaning and it describes an indeterminate structure which maintains its shape and transmits tension in the extent in which it is capable of resisting bending moment. It is well understood that the ectoplasm is not absolutely rigid but has also some elasticity and plasticity (Yagi 1961), as well as the endoplasm has the flow properties of a viscoelastic fluid (Allen and Roslansky 1959).

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cultures and usually considered as "normal". The monopodial forms with prominent watery frontal caps which spontaneously develop in cultures, the monopodial forms without watery caps induced by unidirectional external stimulation, and the forms said "radial" with very long pseudopodia and weak attachment to the substrate produced by sudden changes of the medium, were also recorded, but the analysis of their locomotion is beyond the scope of the present study.

For observation, filming, and photographic recording in the horizontal plane a few glass spheres about 0.7 mm in diameter were added to the sample to keep the necessary distance between the microscopic slide and the cover glass.

Pictures of amoebae in vertical plane were taken with two techniques based on different principles. In the first procedure which was a modification of the classical method of Dellinger (1906), a vertical glass chamber 1.2 mm thick has been used with the microscope mounted horizontally, and in the second, amoebae migrating on the bottom of a crystalization vessel were watched through a system of two mirrors, the microscope being set in its usual vertical position. The details of techniques developed for studying amoebae and other protozoa in the side-view are to be described elsewhere (Grębecki, unpublished).

The macrostructural aspect of the autotraction of the cell frame, expressed by changing the position of the whole contour of moving amoeba, has been recorded both cinematographically and photographically.

The filming technique was essentially the same as used before by Grębecka and Grębecki (1975), and consisted in taking time-lapse sequences at the frequency of 1/s (0.2 s exposure + 0.8 s interval) with a Bolex H 16 Reflex camera steered by the Paillard-Wild Variotimer device and mounted on an interference contrast microscope (power 50 ×). For further analysis, the profiles of migrating amoeba, with some reference points on the substrate, were redrawn on the tracing paper to represent the stages of locomotion spaced at 10 s intervals.

The changing positions of the contours of moving amoebae were also recorded photographically with the double-exposure technique. This is an adaptation of the method developed originally by Doroszewski (1961) for recording motion of the ciliate *Dileptus*. It is based on taking two pictures of the same object, at a known time interval, on the same plate. There should be a difference in the luminosity or in the duration of both exposures, to discern between the initial and the final positions of the moving animal. In this study the double-exposure pictures were taken with a dark field microscope which gives much better contrast of both superimposed contours, than the original bright field method of Doroszewski. The difference between two successive images was obtained by introducing a filter absorbing 75% of light, during one or another exposure. The first exposure should be weaker than the second one to record clearly the withdrawal of a structure (the posterior part of amoeba), and the reverse relation of the light intensities produces good pictures of the advancing part of the cell (the front of amoeba). The exposure time was 0.2 s in each case, with the interval of 20 s in between.

The movement of granules constituent of the ectoplasmic tube, which reveals the autotraction of the cell frame under its microstructural aspect, has been recorded by long-exposure photography, in the dark field. On such records the moving granules produce streaks and the resting ones are seen as spots. The technique was very similar to that used first for amoebae by Rinaldi (1963). In the present study pictures were taken with a low power dark field microscope (magnification 80 × or 120 ×) and on large size photographic plates (6 × 6 cm.), which allowed to record the motion of whole amoebae. The choice of the exposure time is decisive because it affects the recorded pattern and may influence the interpretation. It

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was 20 s in this study, i.e., 3-5 times longer than that used by Rinaldi (obviously, with a longer exposure the light intensity must be kept lower). The longer recording time is necessary to put in evidence the existence of very slow movements of the ectoplasmic granules. On the other hand, with a weak luminosity and long exposure, very fast endoplasmic granules do not produce any streaks at all, but only a gray halo, which excludes confusion between the motion of the ectoplasmic tube and the endoplasmic streaming.

Expectations from the Model

The character of the co-axial movements of the semi-rigid frame of amoeba cell has been in fact in large extent predicted by earlier results obtained in this laboratory (Grębecka and Grębecki 1975). A planimetric study of different body regions of a moving *Amoeba proteus* resulted in designing a pair of models of an "ideal amoeba" in two successive stages of its locomotion separated by 10 s interval (Fig. 1). The

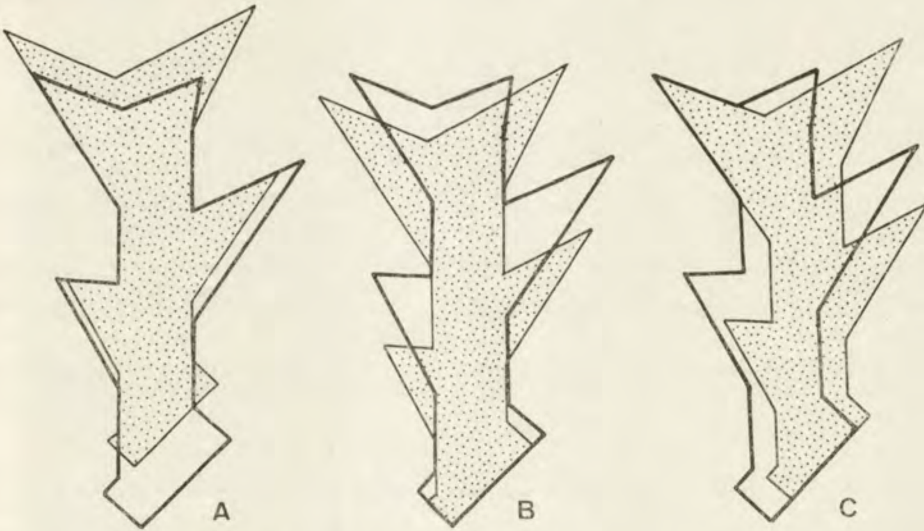

Fig. 1. Graphic model of the "ideal amoeba" in the initial stage of its movement (empty contour), and 10 s later (dotted contour), after Grębecka and Grębecki (1975); both stages are superimposed to show changing of the position of the model, depending on different sites of attachment: A — attachment between the middle and the anterior sub-regions of the trunk, B — attachment by the rear edge of the uroid, C — attachment at the tip of a growing frontal pseudopodium

models express the relative size of all body regions and their changes during 10 s, in particular the reduction of the uroid by 39%, of the contracting pseudopodia by 34%, of the posterior part of the trunk by 35%, and of its middle part by 19%, as well as the expansion of the anterior

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part of the trunk by 9%, and the expansion of the advancing pseudopodia which reaches 31% at their bases and 87% in their free parts.

It is enough to realize that all body regions continuously either shorten or expand, for concluding that the presumed stationary condition of the ectoplasmic tube is physically impossible. All segments of the semi-rigid frame of the cell must change their relative positions simply because they all change their dimensions. What kind of effective movement in respect to the substrate will result from this relative motion, is evidently depending on the position of the sites of attachment of amoeba.

As well our own unpublished observations (Pl. I 1), as earlier descriptions by Dellinger (1906), Bell and Jeon (1963) and Haberey (1971) show that a polypodial *Amoeba proteus* "stands" on a few supporting knobs often developed as to form short pseudopodia. Some of them slide against the substrate and some remain stationary for a certain time. Only the stationary ones may be the structure holding the adjacent portion of the semi-rigid cell frame in a temporarily fixed position. The holding knobs or pseudopodia may be occasionally seen in any place on the under side of amoeba, but most frequently they are found in the region distant, roughly speaking, by $^1/_3$ of the body length from the front, and $^2/_3$ from the tail.

In the Figure 1 A two stages of the "ideal amoeba" taken at a 10 s interval, are superimposed in the manner to keep in the unchanged position the imaginary borderline between the anterior $^1/_3$ and the posterior $^2/_3$ of the body. This corresponds morphologically to the line separating the anterior part of the trunk from its middle part and from the contracting pseudopodia. As a consequence, in this state all contracting body regions are situated behind the zone kept in the stationary conditions, and all expanding regions in front of it (according to the data on the contraction / expansion gradient in amoeba as established before: Grębecka and Grębecki 1975).

Predictions of the model presented in the Fig. 1 A are extremely clear. The semi-rigid cell frame behind the attachement zone is expected to move forwards and the speed of its successive segments should decrease from the tail towards the attachment zone. Also in front of the attachment zone the cell frame will presumably advance and the speed of its movement should be higher for the segments situated more frontally.

Figures 1 B and 1 C present the "behaviour of the model" in two other, and extreme, hypothetical situations: amoeba attached to the substrate at the rear edge of the uroid or at the tip of a frontal pseudopodium. They will be confronted with the actual behaviour of living amoebae further in the text.

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Pulling Effects behind the Attachment Zone

Figure 2 gives some examples of the actual behaviour of the uroid and of the adjacent posterior part of the trunk as recorded in the time-lapse pictures taken in the horizontal plane. Three sequences are drawn, recorded in three different individuals, composed respectively of 12, 7 and 6 successive stages of movement separated each by 10 s intervals.

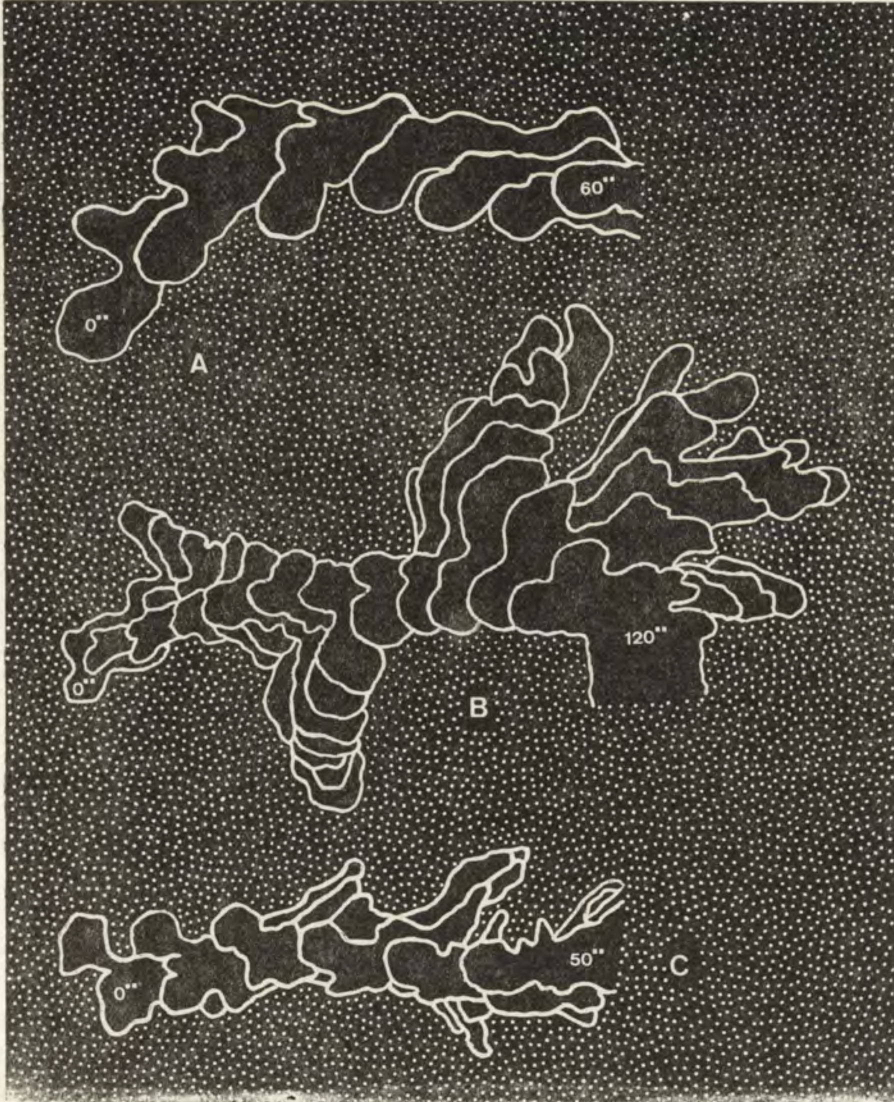

Fig. 2. Examples of dragging forwards of the posterior parts of amoebae filmed in the horizontal plane; all superimposed contours are separated by 10 s intervals

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Some more examples of autotraction of the posterior part of the semi-rigid cell frame of amoeba, obtained with the double-exposure photographic technique, taken at the intervals of 20 s, are shown in the Pl. I 2, 3 and II 4–7.

It is evident from all these pictures that in the posterior part of amoeba not only a contraction takes place, but also a forcible displacement of the contracting structures. All distinct projections of the uroid and small foldings visible on its contour considerably move forwards before they disappear. One could also describe this phenomenon by stating that the shape of the posterior part of amoeba is much more stable than its position in respect to the substrate.

The side-viewing of the posterior part of amoeba in the vertical plane provides essentially similar pictures of dragging of the whole structure towards the zone of attachment, i. e., in the forward direction. A new element seen with this technique is the vertical component of movements of the uroid which may be winded up (Fig. 3 A) and alternately fall

Fig. 3. Example of dragging forwards of the posterior part of an amoeba filmed in the vertical plane, from the side: A — winding up of the uroid during first 20 s, B — falling down of the uroid during the next 30 s

down (Fig. 3 B) in the same individual. After the falling down phase the uroid may touch the ground but usually it is only just loosely lying on it and freely glides on the substrate when it is still drawn forwards. This means that the uroid in this state may constitute a point of support but not a point of attachment for the cell. In general, the observation in ver-

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tical plane confirms that the cell may be attached to the substrate at its rear pole only occasionally and for a short time.

The contracting pseudopodia also, if they keep contact with substrate, usually cannot represent more than the supporting points, because they slide on the ground, when the structure contracts and moves forwards as a whole. Such a case is shown on the Fig. 4 representing three stages

Fig. 4. Sliding against the substrate of the contracting pseudopodia, which support the uroid, in a specimen filmed in the vertical plane, from behind (intervals of 20 s between the contours)

of movement separated by 20 s intervals, in an individual recorded in the vertical plane not from the side but from behind (the amoeba moves away from the observer's eye). The uroid, seen in the upper right part of the picture, is supported but not fixed by two contracting pseudopodia which touch the ground and slide on it.

It has been predicted by the model in the Fig. 1 A that the movement of the cell frame should progressively slow down in its successive segments from the tail towards the attachment zone. The exact measures remain to be taken in future, but in general the ciné-recordings demonstrate that this assumption is also true. Figure 5 shows, in the horizontal plane, three stages of movement of the posterior part of an amoeba which had the uroid in form of a pseudopodial residue and two other contracting pseudopodia were arranged in line on the same side of the body. It is clearly seen that the forward movement of the uroid was the fastest, it was distinctly slower in the case of the first pseudopodium next to the uroid, and the slowest for the second lateral pseudopodium approaching the middle region of amoeba. Also all the double-exposure photo-records shown in the Pl. I and II confirm that the speed of dragging forwards of the posterior part of the cell frame gradually falls down from the uroid towards the middle of the body. This phenomenon is particularly

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Fig. 5. Decreasing rate of pulling forward of the uroid and of two linearly arranged contracting pseudopodia in the posterior part of an amoeba, filmed horizontally

well expressed in three lateral pseudopodia of the amoeba shown in the Pl. II 4.

It may be also seen from all time-lapse ciné-records presented here (Figs. 2–5) that the forward movement of the uroid seems to be sustained and rather stable for considerable periods of time in each individual. This is probably related to the semi-permanent character of the uroid as reported by Goldacre (1956).

Pushing Effects in front of the Attachment Zone

Movement forwards of the anterior part of amoeba's cell frame, between the attachment points and the tips of advancing pseudopodia, is expected from the model (Fig. 1 A) as a summarized effect of the expansion of all body sub-regions in this zone. It is less easy to demonstrate this, apparently for two main reasons. First difficulty is a possible confusion between the expected effect of pushing forwards of the whole structure and the well known phenomenon of its increasing in the pseudopodial tips from the endoplasmic material. For instance, the double-exposure records of the anterior part of moving amoeba (Pl. III 8–9) show only the changing position of the advancing front, but it is impossible to conclude from these pictures how much of the change is due to the apical addition of new segments to the ectoplasmic tube, and how much

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(if any) to the elongation and pushing forwards of the pre-existing structure. Second difficulty consists in that the longitudinal body axis of a polypodial amoeba is more branched in the anterior than in the posterior body part.

The long-exposure photographic technique helps to overcome these difficulties and it puts in evidence the advancing of the free anterior part of the cell frame. Pictures of frontal pseudopodia shown in the Pl. III 10–12 and IV 13–15, taken with a single exposure of 20 s each, demonstrate that the whole ectoplasmic tube in this region of migrating amoeba is slightly pushed forwards: all granules are moving and produce streaks on the photographic plate. A stationary condition of the ectoplasmic tube, represented by resting granules recorded as spots, may be also found frequently but in the basis of a pseudopodium (Pl. III 10 and IV 13–14).

Another manner of discerning the forward movement of the anterior part of the whole cell frame may be based on the observation of changing position of such sections of the frontal edge of moving amoeba which are not supposed to grow on by the addition of new ectoplasm. This is the case of areas from which the bases of two neighbouring advancing pseudopodia emerge, which for the purpose of convenience and in relation to their shape, will be called Y junctions. The behaviour of the Y junctions has been analysed on the time-lapse film records, and some examples of them are given in the Fig. 6 in which were superimposed respectively 4,5 and 5 contours of the advancing front of amoebae, separated by 10 s intervals. In all cases shown in the drawings, the Y junctions (indicated with arrows) change their position in the direction conform with the cell locomotion. It should be noted that this is not occurring every time in all advancing pseudopodia, but it is a very common phenomenon. However, this is sufficient as evidence, because any forward movement of the Y junctions cannot be predicted from the endoplasm-ectoplasm conversion taking place close to the tips of advancing pseudopodia, and is therefore incompatible with the concept of a stationary gel tube in amoeba.

Time-lapse records of the frontal edge of migrating amoebae, taken in the vertical plane, display essentially similar pattern. Examples given in the Fig. 7 put in evidence the forward pushing of Y junctions as seen from the side, and on the other hand, they demonstrate another meaningful fact that the sites in the front of an advancing pseudopodium which establish a contact with the substrate, may not adhere to the glass immediately after touching it, but instead they are most frequently pushed forwards and glide against the bottom before they become the fixed points of attachment.

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Fig. 6. Examples of changing position of the advancing anterior part of amoebae, filmed in the horizontal plane; arrows indicate pushing forwards in the Y junctions between growing pseudopodia

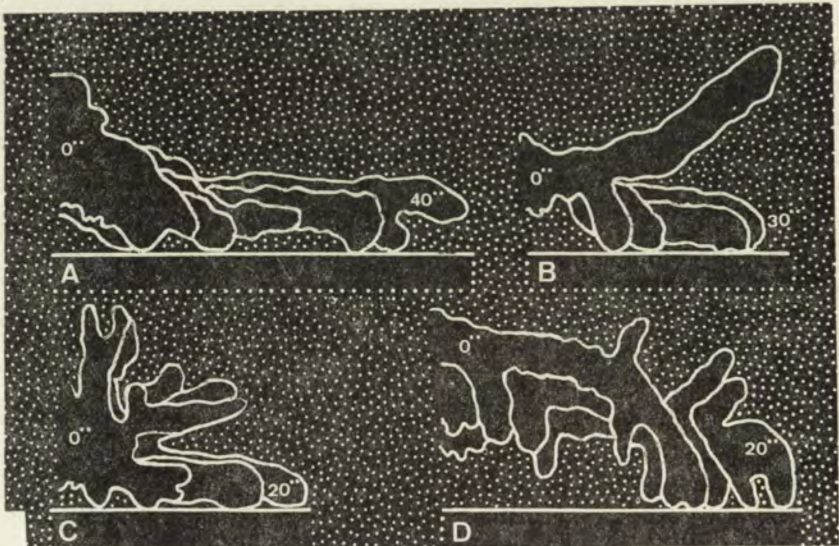

Fig. 7. Examples of the forward motion of the anterior parts of the contours of amoebae, recorded in the side-view

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Crawling Forwards of the Whole Cell Frame

It is impossible to use the double-exposure photographic recording to visualize the forward movement of the semi-rigid cell frame in the whole extent of a migrating amoeba: with the first exposure being weaker than the second one, changing of the position of the advancing front is not recorded, and with the first exposure set stronger, on the contrary, the image of the withdrawed tail becomes very confused. The only manner to obtain an image of the moving cell frame in the whole amoeba on a single still picture, is to record the displacement of the ectoplasmic granules with the long-exposure technique. A choice of examples of such records (taken during 20 s each) is given on the Pl. IV–IX. Regardless of the very different shape of individuals, and of various degree of their polypodiality, being rather small in amoebae represented on the Pl. IV 16 and V 17 and high on the pictures numbered 18–25 on the Pl. V–IX, they all present some common features. It should be noted first that in all cases one cannot find any resting granules, recorded as spots, neither in the posterior half of the body nor in its smaller anterior part, which confirms the pulling of the cell frame behind the attachment zone, and its pushing in front of it, as described in two preceding chapters. On the contrary, the immobile granules indicating a temporary fixation of the ectoplasmic tube are found, as a rule in all pictures, only in a limited middle-anterior body region. This corresponds well to the position of the attachment zone which has been defined here from the side-view pictures as being most frequently located at the distance of $^1/_3$ of the body length from the front and $^2/_3$ from the tail.

It should be added that in highly polypodial animals two or more temporary stationary zones, in two or more branches of the trunk, may be sometimes seen instead of a single one, but their positions still follow the general topography of the attachment sites as described before. A good example of such two separate stationary zones is given by the Pl. V 18.

The amoeba shown in the Pl. IX 26 represents a lobose form with broad and flattened pseudopodia. In such individual the stationary segment of the ectoplasmic tube is much more extended in both directions, but even in this case a forward movement of the cell frame is recorded in the tail region and close to the advancing front.

The macrostructural aspect of autotraction of the cell frame in the whole amoeba may be put in evidence by superimposing two complete contours taken from the time-lapse cinematographic records. Some examples are given in the Fig. 8, in the horizontal plane, and some others in the vertical plane, in the Fig. 9. They show that the lateral pseudo-

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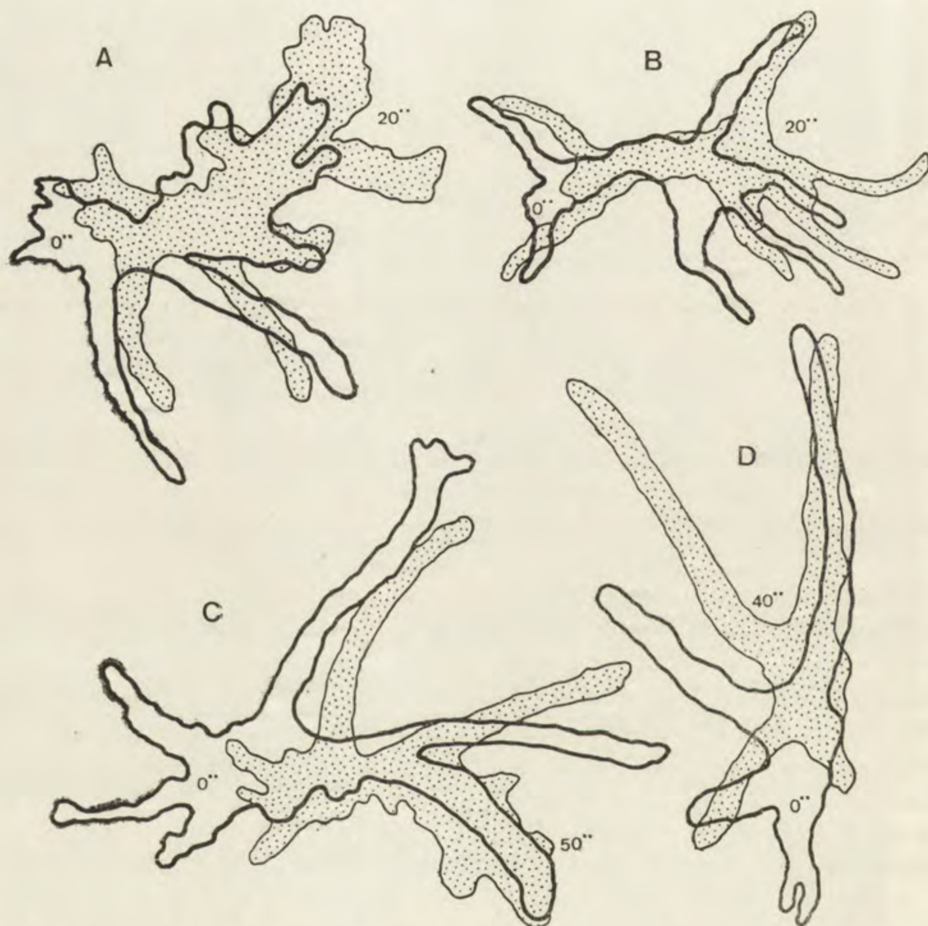

Fig. 8. Examples of the forward motion of the whole cell frame in amoebae normally attached, filmed in the horizontal plane

podia and other identifiable projections of the contours move forwards as well in the posterior part of a migration amoeba as in its anterior part, excepted the zone which is close to the attachment points.

Taking into account that the adhering knobs are not stable, that the posterior ones gradually loose their contact with the substrate, and simultaneously new attachments are being established more frontally, we have to conclude that the ectoplasmic tube is stationary only in a very limited segment, which moreover, is continuously displaced forwards as well in respect to the outer reference points as in respect to the constituent structures of the ectoplasmic tube (Fig. 17). With such changes of position of the stationary attachment zone, pulling in the posterior part of the

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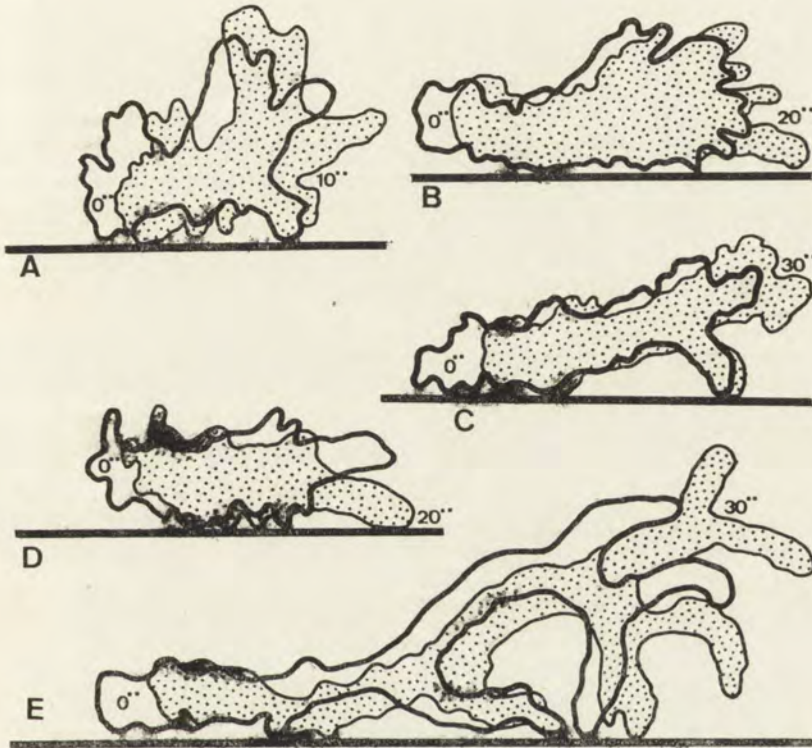

Fig. 9. Examples of the forward motion of the whole cell frame in amoebae recorded in the vertical plane; note the stable position of the middle-anterior attachment sites and gliding forwards of the points of support located in the posterior body regions

ectoplasmic tube, and pushing in its anterior part, must result in a true crawling movement of the whole semi-rigid cell frame of migrating amoeba.

Tail Attachment, Frontal Attachment and Crawling Backwards

As it has been stated before, adhesion to the substrate between the middle and the anterior sub-regions of the trunk is the most common, but not unique manner of attachment of amoeba. Let us consider now two other extreme cases: the attachment zone temporarily situated on the under side of the uroid, or at the tip of a frontal growing pseudopodium.

The movement of the cell frame predicted in both these cases by the model may be read out from the Fig. 1 B and C. First evident conclusion is that an amoeba attached by the tail or exactly by the extreme frontal point cannot actually advance. The tail fixed in one position keeps the

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front also in a stationary condition, and vice versa, assuming that the length of the cell does not change. However, an amoeba prevented in this way to change effectively its position, is still performing movements: the ectoplasmic tube shortens and disintegrates in its posterior part, whereas in its anterior part it expands and new material from the endoplasmic stream is added to it at the tips of frontal pseudopodia. The obvious result, shown by the Fig. 1 B and C, is that such amoeba which cannot effectively move forwards, should manifest a non-effective movement backwards of the ectoplasmic tube and of all its lateral projections, i. e., a kind of crawling backwards of the whole cell frame.

In the time-lapse sequences of locomotion recorded for this study one can find many examples of behaviour fitting very well the theoretical expectations presented above. In amoebae filmed in the horizontal plane one cannot actually see the points of their attachment to the substrate, but it is possible to find specimens with the uroid temporarily immobilized, and on the contrary, individuals with a frontal pseudopodium visibly growing on but immobilized at its tip. Figure 10 provides an example of

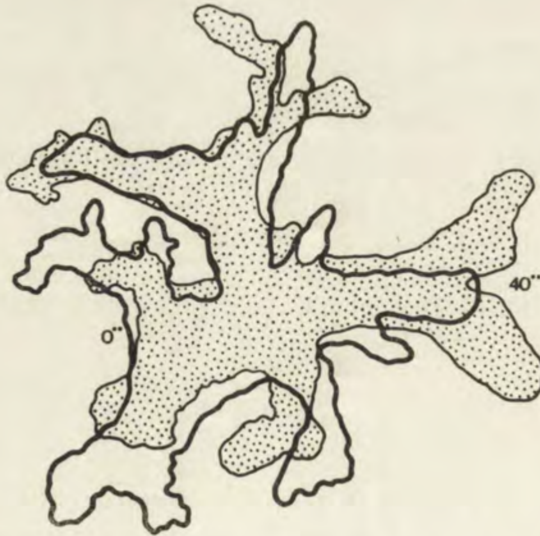

Fig. 10. Backward motion of the whole cell frame in an amoeba, filmed horizontally, in which the uroid was keeping an approximately fixed position

amoeba in which the cell frame moves back being pulled towards the stationary uroid, and the Fig. 11 shows a specimen which is as a whole pushed backwards from the immobilized tip of a growing frontal pseudopodium. It should be stressed that the locomotion is stopped and the cell frame is pushed backward only in the case when the frontal pseudopo-

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dium is touching the bottom exactly with its extreme point, i.e., when its growing tip exerts a force directed against the substrate. Otherwise, if the contact between the frontal pseudopodium and the glass is established more laterally, the growing tip is free to advance, and the locomotion may continue in the usual way, as shown in the Fig. 7.

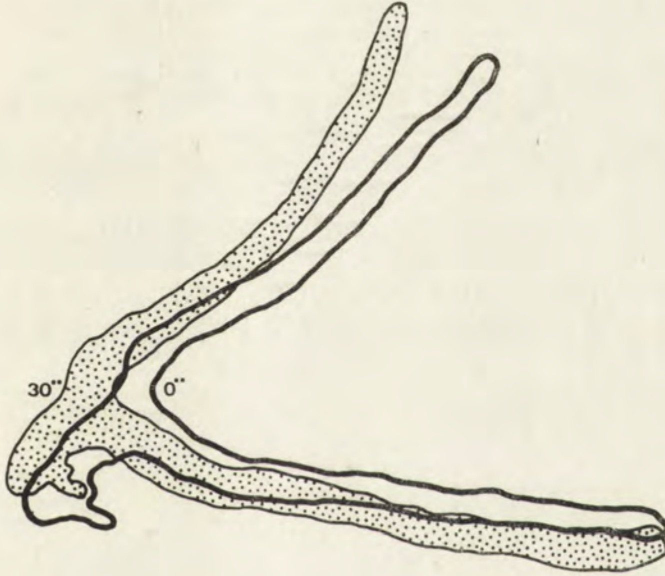

Fig. 11. Backward motion of the whole cell frame in an amoeba, filmed horizontally, in which the tip of the growing frontal pseudopodium was keeping an approximately fixed position

This phenomenon may be recorded also with the double-exposure photographic technique, as well in the whole animal (Pl. X 27) as in a branched frontal pseudopodium (Pl. X 28). In both pictures the weaker contours correspond to the initial position of amoeba, and arrows indicate the tips of pseudopodia which keep fixed positions in spite of their growth.

In side-view (Fig. 12) the crawling backwards of the cell frame is

Fig. 12. Examples of backward motion of the whole cell frame in amoebae attached in the manner preventing the effective locomotion, as recorded in the side-view

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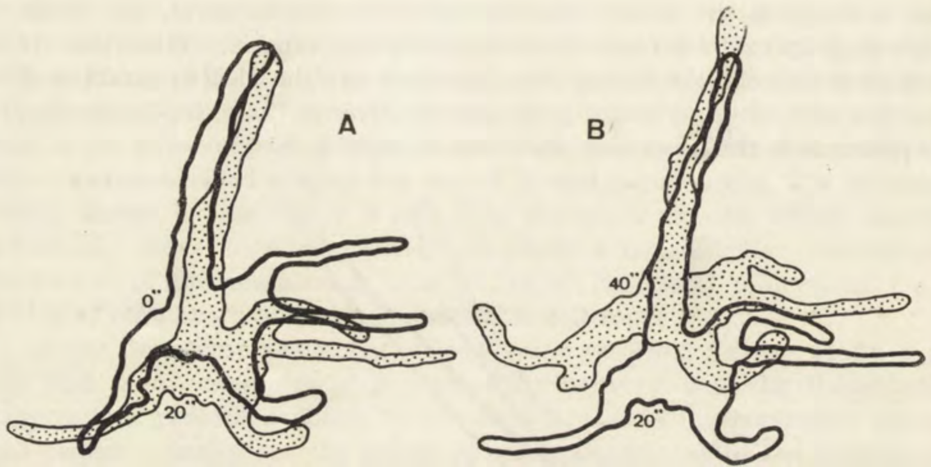

Fig. 13. Backward motion of the whole cell frame, without effective locomotion, in a specimen fixed, during the first 20 s, at the tip of its frontal pseudopodium (A), and the recovery of locomotion and of the forward movement of the cell frame in the same individual, during the next 20 s (B)

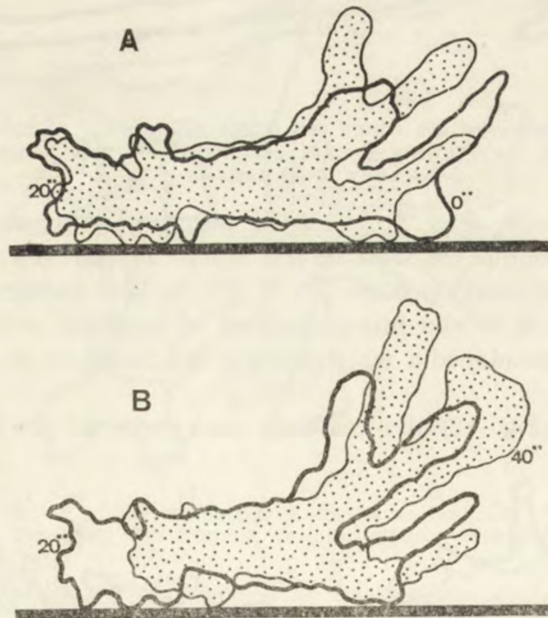

Fig. 14. Side-view picture of an amoeba attached by the uroid, for the first 20 s (A), and re-establishing normal attachment in the next period (B); the absence of effective locomotion and the backward motion of the whole cell frame are correlated with the tail attachment, whereas the middle-anterior attachment results in active locomotion with the cell frame crawling forwards

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equally well pronounced and it may be confronted with the fixed or changing position of the supporting knobs and pseudopodia.

The crawling backwards of the cell frame in amoeba attached at its rear or at its front, is not a phenomenon lasting for a long time, and it is followed by establishment of new attachment points in the middle-anterior body region. This results in restoration of the effective locomotion accompanied by the usual behaviour of the cell frame. Figure 13 shows a case of amoeba, filmed in the horizontal plane, which has been attached at the front for the first 20 s of recording, and recovered the normal locomotion during the next 20 s. In the Figure 14 an example is given of another specimen, filmed from the side, which initially has been fixed to the substrate by the uroid, and also began to move normally in the next 20 s period. In both cases, when the effective locomotion is stopped, the whole cell frame moves backwards, and when the cell starts to advance again, the cell frame immediately resumes its crawling forwards.

Discussion

The autotraction of the semi-rigid cell frame in *Amoeba proteus* is a phenomenon so regular, that in fact it has been several times recorded in some earlier studies, but seldom clearly shown on illustrations and never recognized as one of the fundamental aspects of cell locomotion to be thoroughly investigated and incorporated into a comprehensive theory of amoeboid movement.

For example, basing on the time-lapse recordings published by Stockem et al. (1969), it is easy to put in evidence the movement forwards of the cell frame, provided that the original sketches are redrawn in the manner adopted in this paper. The Figure 15 brings the results of such exercise as applied to the Fig. 4: stage I of the discussed article. The Figure 15 B in which both complete profiles, separated by 60 s. interval, are superimposed, shows exactly the same pattern of behaviour of the cell frame as described in the present study. This pattern is obscured in the original sketch (Fig. 15 A) because of confusion between these parts of the contour which undergo a real change of dimensions, and those which simply change their position. For instance, the black colour is used both for the growing part of frontal pseudopodium and for some areas of the uroid which does not grow but moves forwards as a whole.

This confusing interpretation results from referring to the substrate only, and not to amoeba body itself, when analyzing the morphological changes between two successive stages of locomotion. This question has been extensively examined in the precedent paper (Grębecka and

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Grębecki 1975) in which we abstained to discuss the numerical data of Stockem et al. (1969) (Table 1), concerning the increments and losses in the contour areas, as being not comparable with our results and not reflecting the size evolution of the separate body regions.

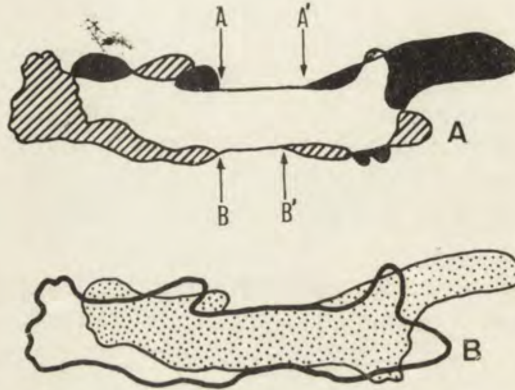

Fig. 15. Forward motion of the cell frame in an amoeba recorded by Stockem, et al. 1969 (Fig. 4: stage I); A — reproduction of the original drawing, B — the same picture redrawn in the manner adopted in the present study

More important is, however, that the time-lapse records of Stockem et al. (1969), when differently graphically presented, confirm the shifting forwards of the cell frame in moving amoeba. It is worth to be pointed out in addition that the region in which these authors found no contour changes (A-A′, B-B′ in the Fig. 15 A), corresponds exactly in its topography to the zone of attachment and of temporary fixation of the cell frame, as described in the present study.

In the ciné-recordings published by Goldacre and Lorch (1950) and republished by Goldacre (1957) (Fig. 1), the autotraction of the cell frame is apparently absent, but different foreign particles visible in the field, which should constitute the fix reference points, are not kept in the same place over the sequence, which proves that the positions of whole pictures have been changed in respect to one another, in filming or in printing. It seems interesting to check if this classical picture really differs from the records obtained is the present study. In the Figure 16 A the original prints of Goldacre are redrawn, to show this difference which completely changes the interpretation. In the Figure 16 B the Goldacre's records, after readjusting their mutual positions in the manner to keep the outer reference points constant, have been graphically represented in the way adopted in the present study. It becomes evident that, after this correction, the Goldacre's amoeba does not manifest the stationary condition of the ectoplasmic tube any

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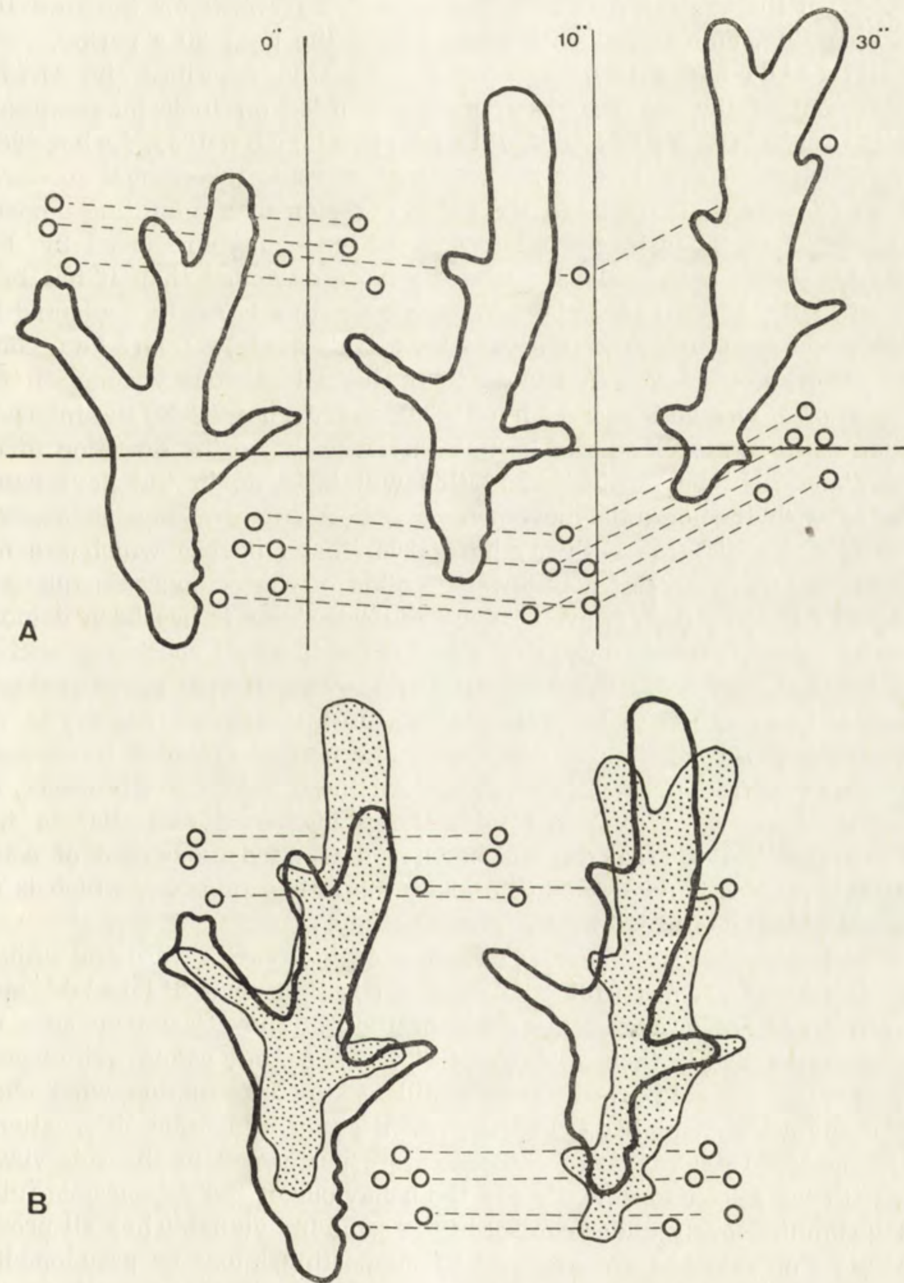

Fig. 16. Three stages of locomotion of amoeba from the time-lapse film frames published by Goldacre (1957) (Fig. 1); A — contours redrawn from the original publication, circles connected with thin lines indicate the position of foreign particles identifiable in the field; B — contours superimposed, and readjusted in manner to keep the external particles in the unchanging positions

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more. On the contrary, its cell frame crawled forward for the first 10 s of recording, and moved backwards during the next 20 s period.

Rinaldi and Jahn are only authors who described the forward movement of the ectoplasmic granules, recorded on their long-exposure "pictographs" (Rinaldi 1963, Rinaldi and Jahn 1963, Jahn 1964), and concluded correctly that the whole ectoplasmic tube moves forwards, as well in the posterior as in the anterior region of a migrating amoeba. The stationary zone in the mid-region of amoeba, as reported by Rinaldi (1963), apparently seems to be more extended than it has been found in the present investigations, however, this is easily explained by very short exposure time (1 s vs. 20 s in this study): Rinaldi could not record slow movements close to the actual stationary zone. In the studies of Rinaldi and Jahn (1963), and Jahn (1964) the interpretation of some movements of granules not parallel to the direction of locomotion might be disputed, and this will be done in the next paper dealing with the non-axial movements of the cell frame of *Amoeba proteus*. One can also disagree with Jahn (1964) that amoebae which are not locomoting and manifest a backward motion of the ectoplasmic tube are blocked "at some point", even "along the sides". As it has been demonstrated in the present study, this effect is produced in specimens anchored by the uroid or by the very tip of a growing frontal pseudopodium, whereas the fixation to the substrate at the intermediate regions is, on the contrary, a *conditio sine qua non* of the normal effective locomotion with the cell frame crawling forwards. All these points are, however, of a minor importance compared with the fundamental fact that in the "pictographs" of Rinaldi and Jahn a forward movement of ectoplasmic granules is recorded in normally migrating amoebae, which is in the full extent confirmed by the present study.

The papers discussed here were subjected to a very severe and unjust criticism by Allen (1973). The opponent is right that Rinaldi and Jahn were not aware of the exact position of the attachment sites of amoebae recorded in their "pictographs", but if so, one cannot reinterpret their results, as Allen did, when still lacking information what character the attachment had [3]. In the present study numerous observations and time-lapse recordings of amoebae were performed in the side-view, i.e., with the attachment points and the behaviour of the ectoplasmic tube being simultaneously under control in the same individual. They all prove that most of amoebae are attached by supporting knobs or pseudopodia located in the middle-anterior region of the body, and that in such speci-

[3] Incidentally, in the Fig. 7 of the paper of Allen 1973 the attachment area is drawn in an oversimplified manner not corresponding to its typical position in respect to the body neither to its actual conformation.

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mens "forward movement of granules shown in the anterior portion of the gel tube demonstrates that the tube is being stretched forward" (Rinaldi and Jan 1963), and not that "the ectoplasm of the anterior two-thirds of the cell remains stationary with respect to the substratum" (Allen 1973).

The discovery of the forward motion of the posterior and anterior portions of the ectoplasmic tube, unfortunately has never been integrated by Rinaldi and Jahn with the general concept of locomotion in amoeba [4]. Integrating point is that the limited stationary zone in the middle-anterior segment of the cell corresponds to the attachment sites, and therefore it changes continuously its position as well in respect to the substrate as in respect to the morphology of amoeba (but usually less in respect to the body length). A scheme explaining the principle of such behaviour is presented in the Fig. 17. The gradual shifting forwards of the stationary attachment zone means that, step by step, at the successive stages of its locomotion, the amoeba becomes anchored to the new sites on the substrate with new supporting knobs, which makes that pulling of the posterior part of its cell frame and pushing of its anterior

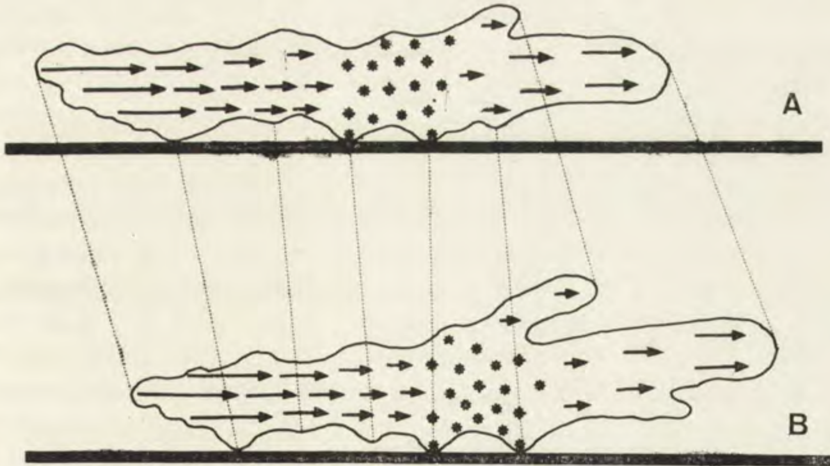

Fig. 17. Schematic representation of the forward crawling of the cell frame of amoeba, as seen from the side, in two successive stages of locomotion (A and B); the arrows of variable length reflect the differentiated rate of the forward motion of the ectoplasmic granules and of the whole contour, and the asterisks indicate the attachment zone in which the cell frame temporarily assumes the stationary condition; dotted lines connect the same morphological points in both successive stages

[4] Schmoller 1971 described "a low rate movement of the hyaline ectoplasm" in *Labirynthula* and generalized this finding by suggesting that it may play some role in the locomotion of giant amoebae, according to the Seravin's idea of multiple mechanisms of ameboid movement (Seravin 1965, 1967).

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portion become locomotory effective and result in a real crawling movement of the whole cell.

The fact that the normally migrating cells usually adhere to the substrate at the zone distant by about $^1/_3$ of the body length from the front and $^2/_3$ from the rear edge, has at least two plausible and complementary explanations, pertaining one to the mechanism and another to the effectiveness of this manner of attachment. It has been demonstrated elsewhere (Grębecka and Grębecki 1975) that the posterior $^2/_3$ of amoeba undergo shortening during locomotion, and the anterior $^1/_3$ is expanding. The imaginary borderline separating both parts is located between the middle and the anterior sub-regions of the trunk. In this area there is very little, if any, shortening or elongation of material between the supporting knobs and pseudopodia, and as a consequence, the mechanism of their adhesion to the substrate is not counteracted by any tension developed by the cell. On the other hand, if all contracting segments which are to be pulled remain behind the attachment zone, and the whole expanding portion which is to be pushed forwards is situated in front of it, the crawling forwards of the cell frame is most vigourous (Fig. 1 A), and it can contribute in the highest extent to changing the position of the whole cell. In other words, amoeba usually attaches itself between the middle and the anterior parts of its body because this manner of adhering to the substrate is the easiest one and the most efficient for locomotion.

It may be extrapolated that the crawling movement of the whole cell frame becomes less efficient if the attachment zone is shifted in either direction, and is temporarily located in the contracting or in the expanding regions of the cell. In the case of attachment at one of the extreme body edges (Fig. 1 B and C), the locomotion becomes completely stopped and the cell frame starts to move backwards to compensate the flow of material transported by the endoplasmic streaming. These events have been described in a similar manner by Jahn (1964), as a consequence of the relativity of motion of body layers in amoeba, and as a mechanism of arising of the so called "fountain streaming".

This question brings us back to some generalizations advanced by Allen (1973). Allen is perfectly right when stating that "in an unattached monopodial cell, the cytoplasm must circulate... in order to satisfy the principle of conservation of mass in a closed system", but this does not necessarily implie that "it is failure to attach to the substratum which typically produces fountain streaming", in particular in polypodial amoebae investigated by Rinaldi and Jahn (1963), Jahn (1964), and in the present study.

In some experiments of the present author (unpublished) the amoebae

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were suspended without contacting the glass, in a viscous medium containing the methylocellulose and in the inuline solutions in which the viscosity is only very slightly altered. As a matter of fact, the ectoplasm of the monopodial forms flows backward in both cases, but the polypodial amoebae stop completely any motion, including the intracellular streaming, in the methylocellulose solutions, and when suspended in the non-viscous medium, they exhibit a variety of different non-axial movements but never a fountain streaming. Also Seravin (1966) was unable to demonstrate the fountain streaming in the unattached polypodial cells. On the contrary, in the present study the impression of a fountain-like flow was produced in all cases of the backward motion of the whole cell frame, i.e., in amoebae temporarily attached at one of the extreme points of their body, most frequently at the front. This is much more in line with the description given to the "fountain streaming" by Jahn (1964). The general conclusion from the present side-view observations and records is that the apparent fountain in the polypodial amoebae is typically produced not by a failure to attach, but by an attachment effected at the very tip of a frontal pseudopodium which makes it unable to advance in spite of its growing on. It should be stressed again that this phenomenon is typical for pseudopodia of polypodial specimens without frontal caps, but the presence of such caps either in the form of native vacuoles (Korohoda and Stockem 1976) or after injection of oil droplets (Grębecka in preparation) produces a fountain-like streaming also in non-attached pseudopodia.

The usual crawling forwards of the cell frame of an amoeba normally attached to the substrate is better pronounced in the body segments which are more distant from the attachment zone. It may be explained by two complementary reasons. The rear edge of the uroid is pulled faster than any other more proximal segment of the contracting part of the trunk, because its forward movement is due to the summarized effects of shortening of all the sub-regions separating it from the attachment sites. The second contributing factor is the longitudinal gradient of the contraction rate (Grębecka and Grębecki 1975). The reasons of the differentiated speed of crawling forwards of the anterior portion of the cell frame look more complicated because their explanation probably depends on the nature and the means of transmission of the forces which are pushing the ectoplasmic tube in front of the attachment zone.

A tentative answer only could be given yet to this last question. The most plausible explanation for the expansion and pushing forwards of the anterior portion of the cell frame seems to be the hydraulic and/or mechanical pressure exerted directly on the tips of advancing pseudopodia by the abutment of the endoplasmic streaming. From the tips, the ten-

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sion would be transmitted backwards through the ectoplasmic tube which should result in stretching it. In the posterior $^2/_3$ of amoeba, the pulling force seems to be transmitted, from the attachment zone up to the rear edge of the uroid, mostly by the ectoplasmic tube itself. However, also in this part of the body some additional transmission of the force through the endoplasm could not be completely excluded, either in the form of a hydraulic under-pressure generated by the outflow of endoplasm or in the form of mechanical dragging accompanying the recruitment of the ectoplasmic elements into the endoplasmic stream.

RÉSUMÉ

La locomotion des amibes était enrégistrée par les techniques de la photographie à l'exposition double et à l'exposition prolongée, ainsi qu'en cinématographie à cadence ralentie. Les observations et les prises de vue se faisaient sur les plans horizontal et vertical (vue de profil). Les individus étudiés restaient tous bien attachés à la surface du fond, et n'étaient soumis à aucune stimulation. L'adhésion à la surface s'effectue le plus souvent par l'intermédiaire des proéminences du tronc ou des pseudopodes localisés sur le côté "ventral" de la cellule, dans une région éloignée approximativement de 1/3 de la longueur du corps du front de l'amibe et de 2/3 de son extrémité postérieure. Cette manière d'attachement se trouve la plus efficace pour la locomotion.

Le tube ectoplasmique n'est pas immobile. Dans les conditions d'adhésion décrites ci-dessus, il subit le raccourcissement, et par conséquence une traction vers l'avant, dans les régions de la cellule situées derrière la zone d'attachement, et devant cette zone il s'allonge ce qui le repousse vers le front. Ce mouvement peut être rěvélé sous la forme du déplacement des granules faisant part du tube ectoplasmique, et sous la forme des changements de position de tout le contour extérieur de l'amibe. L'ectoplasme devient passagèrement immobile seulement pour une brève période, dans la zone assez limitée qui, pour le moment donné, reste attaché à la surface du fond. Cette zone, tout en gardant les distances peu variables qui la séparent du front et de l'uroïde, néanmoins change continuellement sa position, aussi bien par rapport au milieu extérieur que par rapport à la morphologie dynamique de l'amibe. Autrement dit, la cellule s'attache d'un moment à l'autre, dans d'autres endroits du fond et par d'autres proéminences de son corps. Le résultat qui s'ensuit est un véritable mouvement rampant de toute la structure semi-rigide encadrant la cellule, effectué dans la direction conforme avec le sens de la locomotion.

Les individus qu'on trouve sporadiquement attachés par l'uroïde, ou plus souvent, par l'extrémité d'un pseudopode frontal en croissance, sont rendus incapables du mouvement progressif, et pour compenser le courant de leur endoplasme, tout le cadre semi-rigide de la cellule se met à ramper vers l'arrière. L'image qui en résulte produit l'impression d'une circulation du cytoplasme connue dans la littérature sous le nom du courant en fontaine. Le phénomène n'est que temporaire et il est toujours suivi par le re-attachement normal, après quoi le cadre semi-rigide de la cellule reprend son mouvement rampant progressif.

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EXPLANATION OF PLATES I–X

1: Frame from a time-lapse film sequence taken in the side-view through a system of two mirrors. The amoeba migrates freely on the bottom of a cristallization vessel. The upper image shows its real position, and the lower one is its secondary mirror reflection. The cell is touching the substrate in the places in which both images converge. In the specimen shown on the picture, the middle-anterior attachment point has been stable over the sequence, and the uroid was gliding against the glass
2–7: Double-exposure records of the posterior body parts of moving amoebae. The subtle granulated contours represent the initial position, and the overexposed white ones correspond to the position assumed 20 s later
8–9: Double-exposure records of the anterior body parts of two amoebae. The initial contours overexposed
10–15: Movement of ectoplasmic granules in the advancing frontal pseudopodia as recorded with the exposure time of 20 s. Resting granules are seen only on some pictures at the basis of the pseudopodium
16–17: Long-exposure records (20 s) of two complete amoebae showing a low degree of polypodiality. Stationary granules are seen only in a limited middle-anterior zone
18: Two stationary zones arranged in parallel in a specimen dichotomically branched. Beside them, the ectoplasmic granules move forwards, as well in the common tail as in all the advancing pseudopodia
19–25: Long-exposure pictures showing the middle-anterior position of the temporary stationary zones in different polypodial individuals, and the forward motion of the ectoplasmic tube in the more posterior and in the more frontal body regions
26: Large stationary zone, and more limited areas of the forward movement of the ectoplasmic tube in the tail and at the front, in a flattened specimen with broad lobose pseudopodia
27–28: Double-exposure pictures showing the backward motion of the cell frame in the whole individual, and in a branched frontal pseudopodium of another specimen. The granulated contours represent the initial situation, and the white ones were recorded 20 s later. Arrows indicate growing pseudopodia which are keeping an unchanged position of their tips

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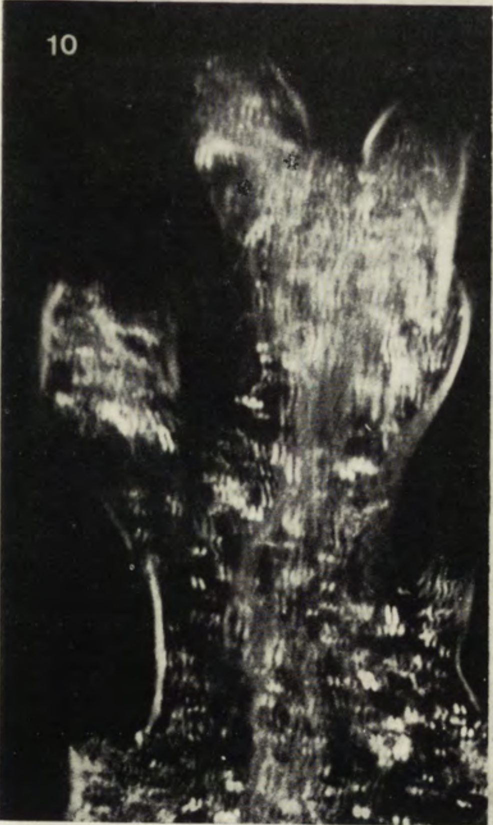

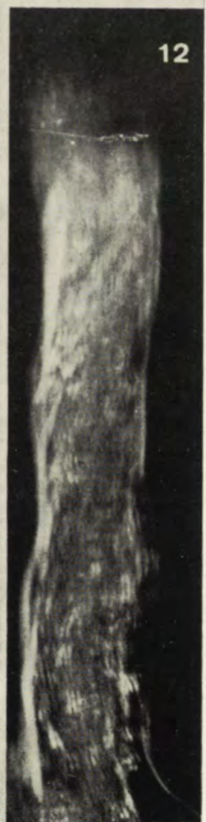

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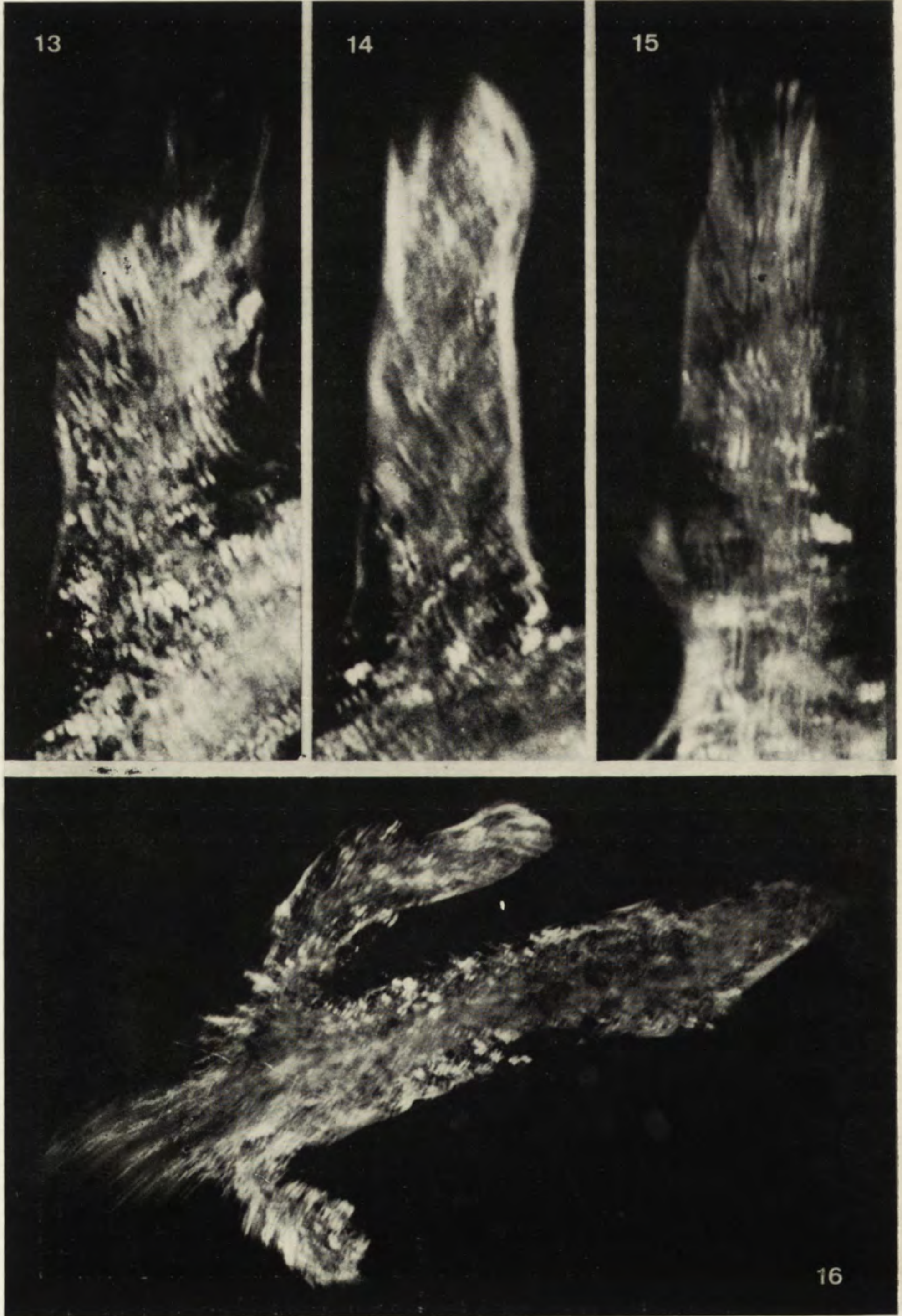

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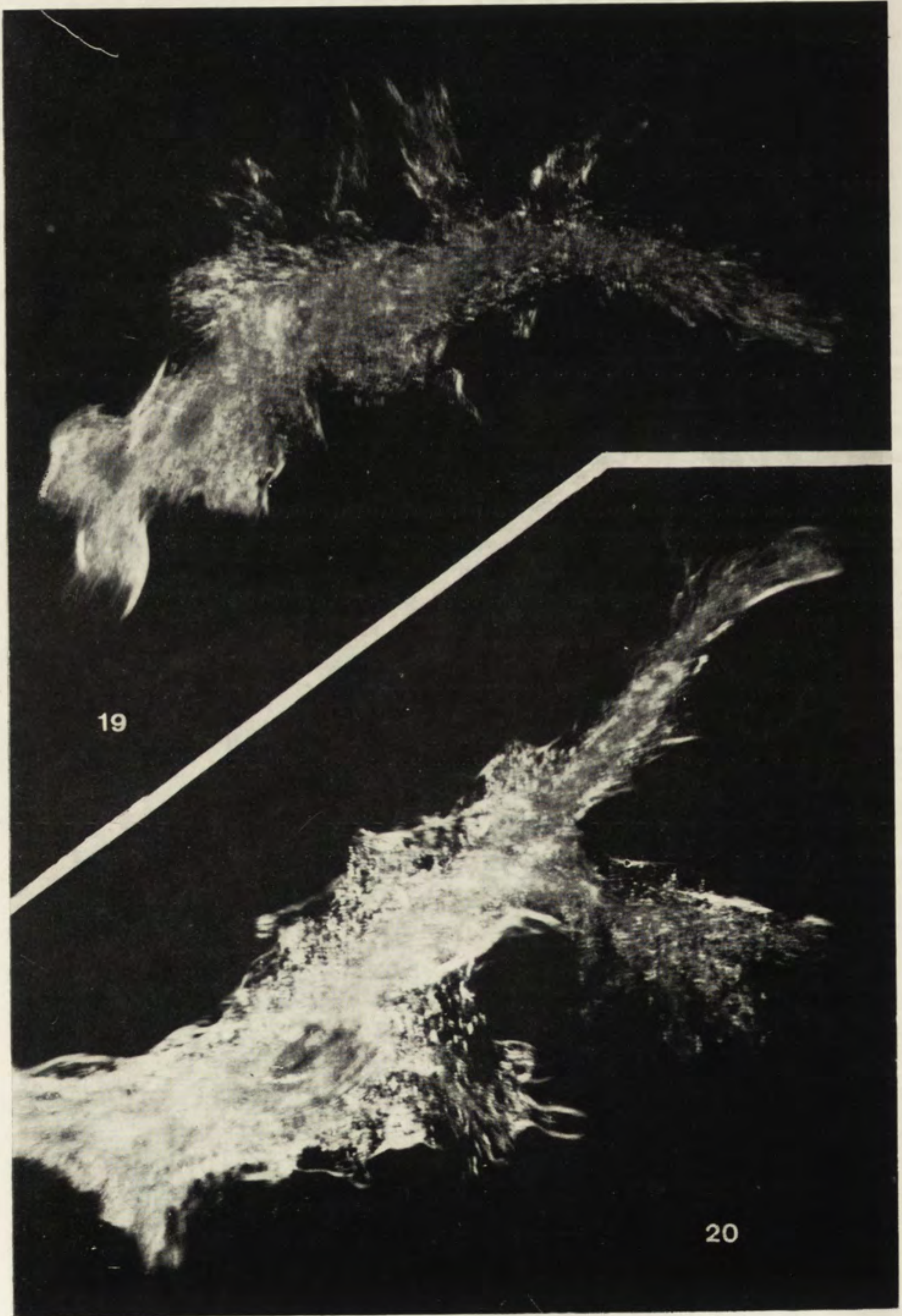

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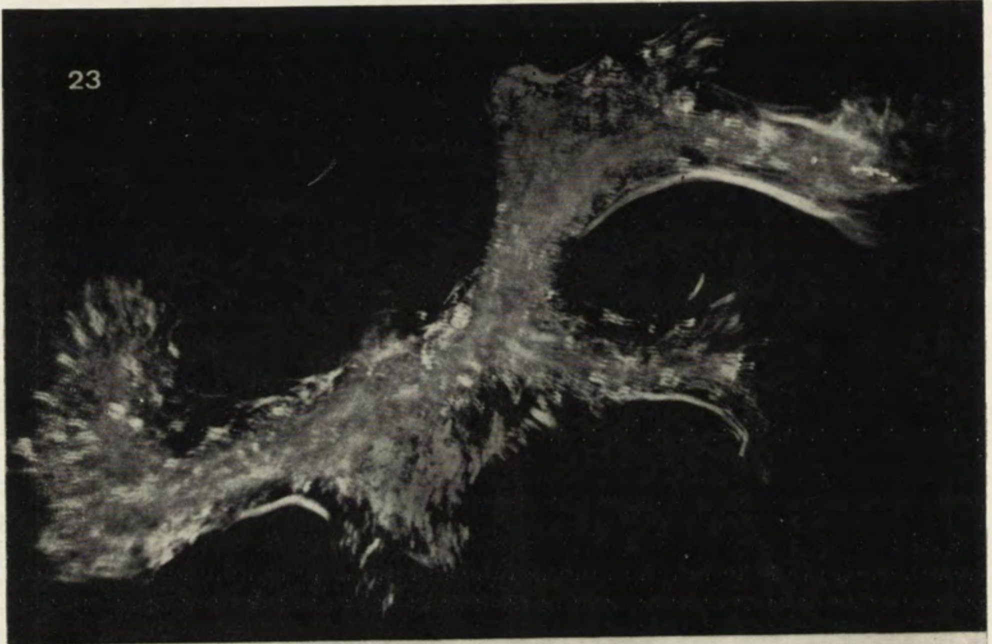

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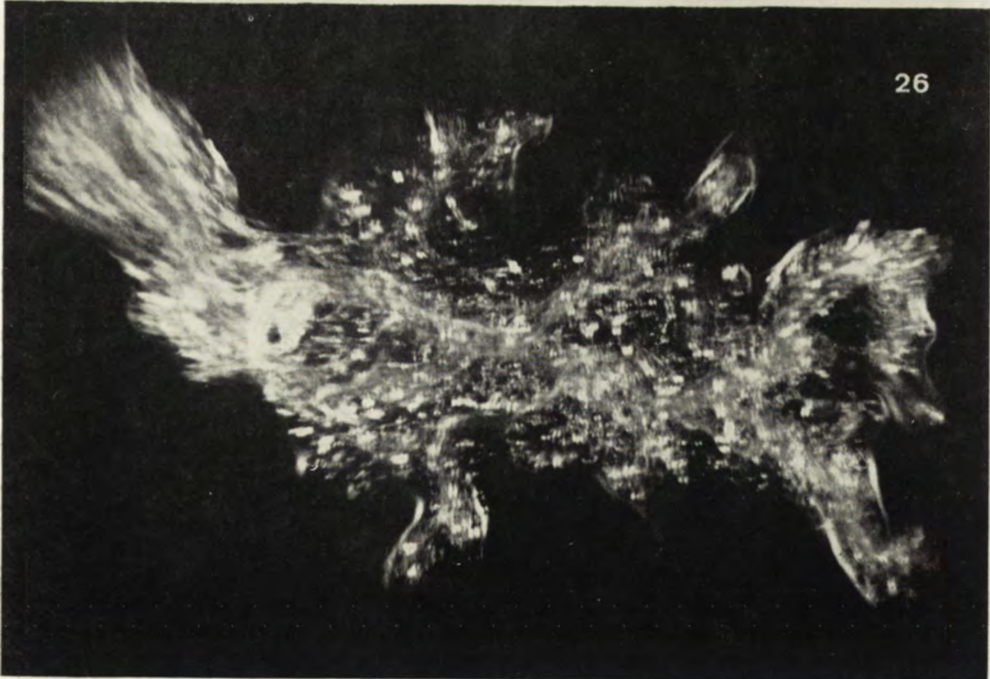

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Motor Response of *Dileptus anser* and *Dileptus anatinus* to Cell Bisection

Synopsis. In *Dileptus anser* and *Dileptus anatinus* approximately 90% of tested animals showed reversal of ciliary beat after cut performed within frontal quarter of the cell, while cell fragments obtained through cut within posterior quarter of the ciliate responded with ciliary beat in normal, backward direction. No preference of any ciliary response could be found in the intermediary region between anterior and posterior quarter of the cell body. The achieved results confirm the occurrence of definite zones of motile reactivity in *Dileptus* exposed to mechanical stimulation.

The problem of localization of motor response areas in ciliates to external stimuli has been since long time an object of interest among protozoologists. It is connected with the important question of postulated occurrence of specific systems of reception to various stimuli in unicellular organisms (Dryl 1974). It is worth to note that as early as Alverdes (1922 a, b) suggested localization of "senses" in protozoa, giving as a proof the differences in observed behaviour of *Paramecium* cell fragments in response to external stimuli. His view was criticized by Koehler (1934) who argued that reactions of intact animals and their fragments showed no essential differences. Holmes (1907) observed ciliary reversal (CR) in *Loxophyllum* as response to cuts performed in the anterior part of the cell body. In extensive studies on response of *Dileptus* cell fragments to mechanical stimuli Doroszewski (1962, 1963 a, b, 1965, 1968, 1972) proved that their anterior fragments react — as a rule — by backward movement i.e., CR, while posterior fragments exposed to water shake or other mechanical stimuli, showed normal forward movement (FM). The aim of the present study is to check the localization of reception areas to mechanical stimulation in those species of *Dileptus* where this problem is less known.

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Material and Methods

The experiments were carried out on the strain of *Dileptus anser* (O. F. M.) and *D. anatinus* Golińska which have been isolated from natural population in the neighbourhood of Warsaw and cultivated during several years in the Department of Cell Biology, M. Nencki Institute of Experimental Biology, Warsaw. The ciliates were kept in Pringsheim's medium and were fed with *Colpidium colpoda.*

In each strain of *Dileptus* 300 microsurgical cuts were performed in various regions of cell by means of a metal microneedle. The animals were placed in a small quantity of Pringsheim's medium (ca. 0.2 ml), surrounded by paraffin oil to avoid drying.

According to terminology introduced by Doroszewski 1963, the term "Backward Response Area" (BRA) was used for the region of cell where the response to cutting appeared as CR, while the term "Forward Response Area" (FRA) was applied to the cell region in which the reaction was manifested as FM. The occurrence of a given reaction in the determined cell region in at least 90% of examined individuals was considered as a criterion for determination of specific zones of reactivity. The cell region which did not show preference for any of above mentioned responses, was called "Intermediary Response Area" (IRA).

Results and Discussion

According to the applied criterion of reactivity in two investigated species of *Dileptus,* three groups of cell fragments could be distinguished (Table 1):

Table 1

Motor Reactions of *Dileptus anser* and *Dileptus anatinus* to Cell Bisection

Group of animals	*Dileptus anser*		*Dileptus anatinus*	
	CR	FM	CR	FM
Group I	91	9	94	6
Group II	62	38	55	45
Group III	5	95	7	93

Group I — 100 animals bisected within' 'Backward Response Area", localized within anterior quarter of cell; Group II — 100 animals bisected within "Indifferent Response Area", localized within two middle quarters of cell; Group III — 100 animals bisected within "Forward Response Area", localized within posterior quarter of cell; CR — Ciliary Reversal Response; FM — Forward Movement Response.

Numbers of operated animals, showing specific motor reactions to cell bisection, are presented in the appropriate columns.

Group I — Cell fragments produced by cuts performed within anterior quarter of animal with reactions characteristic for BRA.

Group II — Cell fragments produced by cuts within two middle quarters of animal with reactions characteristic for IRA.

Group III — Cell fragments produced by cuts within posterior quarter of animal with reactions characteristic for FRA.

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In the group I the backward movement of amputated proboscis and of anterior fragments were of short duration and followed irregular curves, while CR of posterior fragments occurred along more or less regular spiral tracks and lasted much longer. These differences may be connected with a different conductivity of impulses within cell membrane to anterior and posterior pole of the ciliate in some analogy to homodromal and antidromal conductivity in the nerve cells.

The posterior fragments in Group I and II frequently showed not only CR but also nutational movements in form of alternately appearing left- and right-handed spiralling movement. In group III the typical response of both cell fragments to bisection was FM with no CR at all.

The motor reactivity within different regions of bisected cells of two *Dileptus* species is presented in Fig. 1. The zones BRA and FRA proved

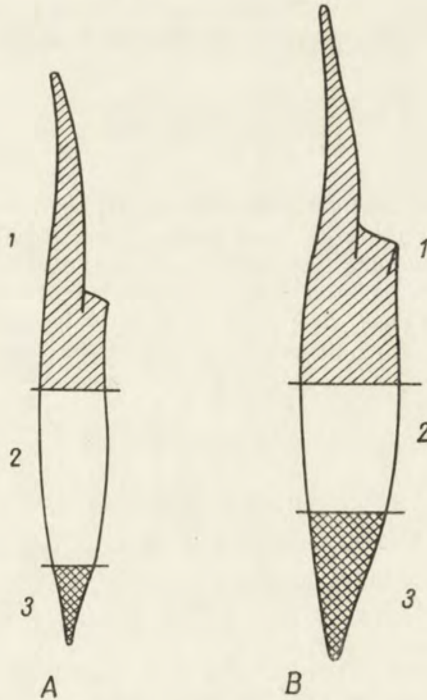

Fig. 1. Localization of motor reactivity to cell bisection in *Dileptus anser* (A) and *Dileptus anatinus* (B). 1 — Backward Response Area, 2 — Intermediary Response Area, 3 — Forward Response Area

to be little more extensive in *Dileptus anser* than in *Dileptus anatinus,* which is characterized by relatively vast area occupied by IRA.

The established motor response areas in *Dileptus* to bisection correspond almost exactly to the analogous response areas observed in non-

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operated animals as concerns their reaction to other mechanical stimuli, like puncture or touching with a microneedle (Doroszewski 1963, Seravin 1963). Both cell fragments produced by microsurgical cuts display analogous reaction and this seems to prove that the sensitiveness to mechanical stimuli characteristic for intact animals, persists initially in the new-formed fragments and that the differences in reactivity within anterior and posterior end of fragment reappear again parallel to post-traumatic regeneration process occurring in the cell body after operation.

It is worth to note that more recently Naitoh and Eckert (1969) also demonstrated differentiation of susceptibility to mechanical stimuli within anterior and posterior ends of *Paramecium*. The mechanical stimulation of anterior region of *Paramecium* evoked CR response which was associated with depolarization of the cell membrane, while more strongly expressed ciliary movement in the normal direction caused by mechanical stimulation of posterior end of *Paramecium* was connected with short-lasting hyperpolarization of the cell membrane.

ZUSAMENFASSUNG

Es wurde festgestellt in *Dileptus anser* und *D. anatinus*, dass nach einem im Bereich des vorderen 1/4 Teiles des Zell Körpers durchgeführten Schnittes 90% der Tiere eine Rückwärtsbewegung vorweisen, während die Zellteile, die nach einem Schnitt im Bereiche des hinteren Körperteiles entstanden — mit einer normalen Vorwärtsbewegung reagierten. Nach einem Schnitt in mittleren Zellteilen keine der angeführten Bewegungsreaktionen bevorzugt wurde. Diese Ergebnisse bestätigen das Vorkommen der bestimmten Zonen der Bewegungsaktivität von *Dileptus* Zelloberfläche als Reaktion auf mechanische Reize.

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