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INTRODUCTION

The symposium "Physiology of motor response in Protozoa" was organized by the Department of General Biology of the M. Nencki Institute of Experimental Biology during the celebrations of Institute's 50-th Anniversary on December 9—12, 1968. It should be emphasized in this respect that the pioneer studies on the physiology of behaviour and an regeneration of the ciliate protozoa were started by the late professor J. Dembowski (1889—1963) and his wife prof. S. Dembowska (1891—1962) soon after establishment of the Institute i.e. after 1918 and were continued before and after the second world-war.

In the last decade the studies on motor response in protozoa constitute the main line of research of the Department of General Biology of the M. Nencki Institute; the extensive experimental work on the response of protozoa to external stimuli (chemotaxis, chemokineses, galvanotaxis, geotaxis etc.), the analysis of mechanism of the ciliary beat in protozoa and application of new, more efficient techniques for recording the movement of protozoa provided the new interesting data for the further development of this kind of research both in Poland and abroad.

The long-lasting tradition and the important role of experimental protozoological studies was stressed recently by the fact that the Department of General Biology of the M. Nencki Institute in Warsaw (with prof. Z. Raabe as a chief Editor) initiated in 1963 the edition of a new international journal ACTA PROTOZOOLOGICA. All the above mentioned facts encouraged the Organizing Committee of Celebrations of the 50-th Anniversary of the M. Nencki Institute of Experimental Biology to organize a separate scientific symposium on "The physiology of motor response of protozoa" as one of four international symposia organized for that occasion.

The symposium includes 9 papers dealing with different physiological aspects of three basic forms of cellular movement of protozoa: ameboid, flagellar and ciliary one.

The author believes that the decision of the Organizing Committee was justified to accept the papers (fasc. 24 and 29) which contribute to our knowledge on the mechanisms of flagellar and ameboid movement, although the experiments were carried out on non-protozoan material. Leszek Kuźnicki's paper "Mechanism of the motor responses of *Paramecium*" will be print in the next volumen of Acta Protozoologica in the considerable enlargement version in comparison with symposium one.

Robert D. ALLEN

Comparative aspects of amoeboid movement

Les aspects comparatifs du mouvement des Amibes

Amoeboid movement is a process of fundamental biological importance because a vast number of different kinds of cells move by means of changes in cell shape, formation of pseudopods, and cytoplasmic streaming. This type of movement is found not only in free-living and parasitic amoebae, but also in many kinds of tissue cells, especially during embryonic development.

On the other hand the variations in the details of amoeboid movement in different kinds of cells is impressive. The best way to grasp the extent of the diversity and to comprehend its meaning is to observe the phenomena first hand. The next best way is to view films, and I have brought with me a ten minute film consisting of short sequences showing five large free-living protists: *Chaos carolinensis, Pelomyxa palustris, Difflugia corona, Actionsphaerium eichorni* and *Allogromia* sp. (strain N. F. Lee) (see Fig. 1).

It is important to recognize that the mechanisms of movement must have evolved in ways that maximized survival in a particular environment and therefore led to efficient feeding, avoidance of predators, and selection of the most favorable physical conditions of light, temperature, gas pressures, etc. Viewed in this context it is perhaps not so surprising that this diversity has arisen. As we shall see, there are almost certainly some basic differences at the cytophysiological and ultrastructural levels among pseudopods of these selected organisms and others that we do not have time to consider here.

The starting point for my own interest in amoeboid movement was the discovery 14 years ago of organized cytoplasmic streaming in naked cytoplasm of the giant carnivorous amoeba, *Chaos carolinensis* (Allen 1955, Allen, Cooledge and Hall 1960). This observation was not consistent with the then "accepted" tail contraction theory of amoeboid movement.

Further examination of the details of movement in intact *Chaos* led to the discovery of a number of further inconsistencies with accepted theory that seemed to suggest the desirability of setting up and testing other models of amoeboid movement (Allen 1961).

Chaos carolinensis, because of its size (up to 2 mm in length), was the material of choice for observation and experiments. Since its movement is carried on by the extension and retraction of pseudopods, we focused our attention on studying the mechanisms of these processes. The first step was to study the details. This was done both by observations of living cells and repeated projection of films. The salient features of cytoplasmic movement
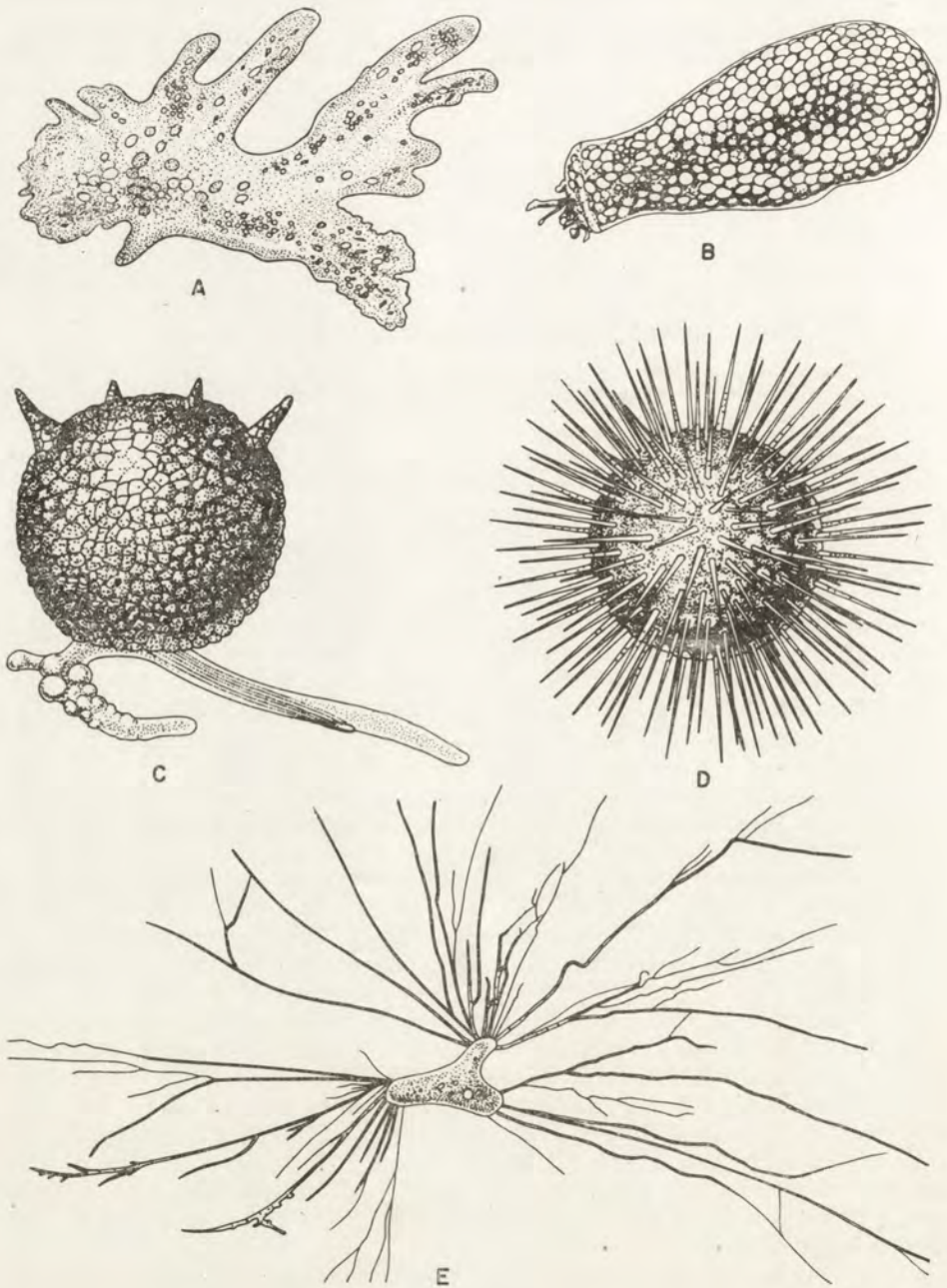
Fig. 1. Five giant sarcodines discussed in this paper: A. *Chaos carolinensis*, B. *Pelomyxa palustris*, C. *Difflugia corona*, D. *Actinosphaerium eichorni* and E. *Allogromia* sp. (strain N. F. Lee)

in pseudopod extension and retraction have been summarized in a recent paper (A l l e n 1968). Some of our findings would have surprised anyone who had noted them prior to the discovery of organized streaming in naked cytoplasm. Perhaps the fact that we were p r e p a r e d to find such phenomena as cytoplasmic counter-currents, forward streaming spurts at the tips of retracting as well as advancing pseudopods, re-reversals initiated at new tips, etc., made the' visualization and objective recording of these events easier (see Table 1). It seems to be easy to overlook phenomena that are not consistent with our preconceptions.

Table 1

A list of features of movement in *Chaos carolinensis* (Modified after A l l e n 1968)

1. Independent pseudopod formation, extension, retraction in polypodial specimens
2. Pseudopods shorten on retraction; endoplasm recruited from walls of ectoplasmic tube
3. Streaming spurts are not synchronized
4. Streamlets move in channels through ectoplasmic tube
5. Spurt accelerations are propagated posteriorly from the tips of pseudopods
6. Waves of acceleration are propagated from tip in reversals and re-reversals
7. Anterior hyaline cap formation precedes spurts, reversals, and re-reversals
8. Backward movement in anterior ectoplasm, when it occurs, represents shortening
9. Three related patterns of streaming exist: fountain, loop, and "roller-towel"
10. Bi-directional streaming and counter-currents
11. Oscillations in quiescent cells, periodic fluctuations in streaming velocity
12. Organized streaming continues in cell-free system showing fountain, loop patterns

In 1961 a model was proposed that was at least consistent with the observed and recorded details of pseudopod formation and retraction (A l l e n 1961 a). The "frontal contraction model" not only fitted the descriptive information and offered a possible explanation of streaming in naked cytoplasm, but it suggested some experiments that might test the basic concept that the force might be frontal contraction forcing the ectoplasm and endoplasms in opposite directions (Fig. 2).
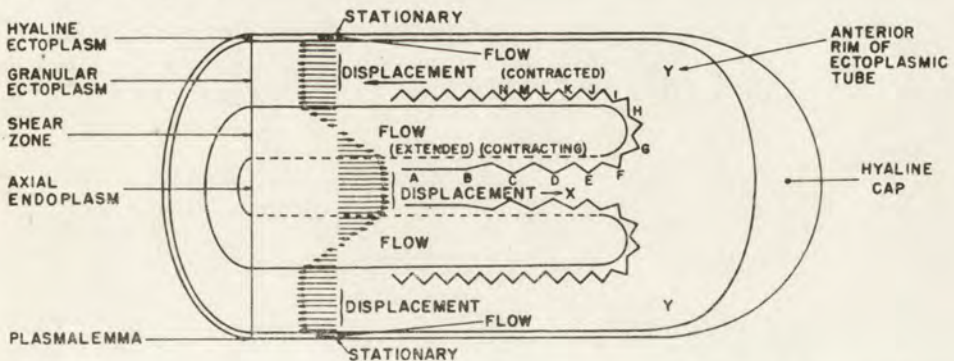

Fig. 2. A diagram of the frontal contraction model of pseudopod extension in *Chaos*

The frontal contraction model required two important tests: First, whether the endoplasm was structurally competent to transmit tension, and second, whether the postulated tensile and compressive forces could be found in the anterior endoplasm and ectoplasm respectively.

The first test received an encouraging but somewhat inconclusive support in rheological studies showing at least pseudoplastic structure in the central part of the endoplasmic stream (A l l e n and R o s l a n s k y 1959, A l l e n 1961 b). More recently, birefringence has been induced in the endoplasm by applying a sharp suck to the tip of a pseudopod. These experiments were carried out in collaboration with Dr. David Francis.

The detection of the motive force for pseudopod extension in *Chaos* was accomplished by using the pseudopod's cytoplasm as a photoelastic strain-guage (A l l e n, F r a n c i s, and N a k a j i m a 1965). A protein gel under tension or compression should exhibit positive or negative birefringence respectively. Birefringence of the two signs is visible as bright and dark contrast between crossed polars with the aid of a weak positive bias phase retardation introduced by a $\lambda/30$ mica (Köhler) compensator (Fig. 3).
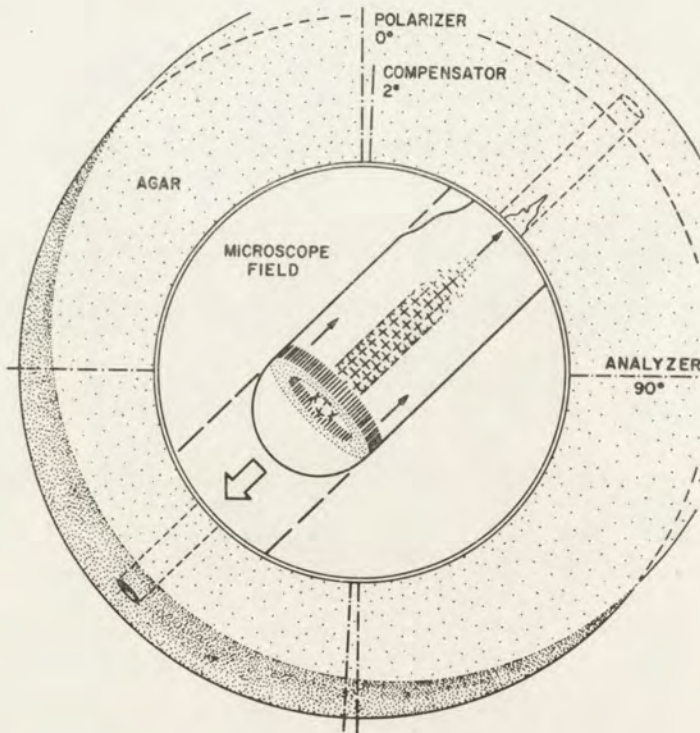

Fig. 3. A diagram summarizing the experimental conditions under which cyclic birefringence changes were recorded from *Chaos* pseudopods exhibiting sporadic flow. For original data, see A l l e n, F r a n c i s and N a k a j i m a 1965

Chaos pseudopods viewed with their axis at $+45$ degrees to the plane of the polarizer most often showed very weak and persistent positive axial birefringence but no cyclic birefringence changes. However, in a few cells exhibiting sporadic streaming, each spurt was accompanied by a "flash" of positive birefringence appearing in the endoplasm just posterior to the hyaline cap. This bright region of positive birefringence then was propagated toward the tail over the forward-streaming endoplasm. At the same time a band of negative birefringence appeared in the anterior ectoplasmic tube and moved backward (relative to the tip) with the ectoplasm. The birefringence was strong at the beginning of each spurt cycle, but weak or absent at the end.

The dependence of the cyclic birefringence on sporadic streaming, its timing with respect to the spurt, and the sign and location of the positive and negative birefringence are all consistent with the interpretation that the birefringent regions of opposite sign represent stretched endoplasm and compressed ectoplasm as proposed in the model. At the present time, the behavioral evidence together with the cyclic birefringence observations present strong evidence supporting the frontal contraction model in *Chaos carolinensis*. It seems probable that the model applies equally well to *Amoeba proteus*, *Amoeba dubia*, and *Amoeba discoides*, where the details of movement differ scarcely at all from those in *Chaos*. These species are less than ideal objects for polarized light studies, however, because of their smaller size and more numerous light-scattering inclusions. All of these large carnivorous amoebae are fast-moving, responsive to stimuli (especially light applied to pseudopod tips) and efficient at capturing prey.

Another giant amoeba is quite the opposite in a number of respects. *Pelomyxa palustris* is an herbivorous species that lives at the bottom of lakes with anaerobic muddy bottoms. It moves by fountain-like cytoplasmic streaming within a single pseudopod of characteristic clubbed shape. The cytoplasmic constituents of *Pelomyxa* are vastly different from *Chaos*. Vesicles abound, as do symbiotic bacteria and sand grains. The ingestion of sand is apparently a mechanism for assuring sedimentation into the soft anaerobic muddy bottom of some lakes.

The environment, nutrition and behavioral requirements of this organism are so different from those of *Chaos* that it is not surprising to find striking differences in the basic features of movement. These were summarized in a table from a paper by G r i f f i n (1964).

The conclusion that can be drawn from these observations is that a tail contraction model seems to be consistent with present behavioral evidence about amoeboid movement in *Pelomyxa*. So far, however, we lack experimental confirmation comparable to that obtained for frontal contraction in *Chaos* by polarized light.

Testaceans represent a third type of amoeboid giant characterized by a heavy shell made of sand grains and detritus. *Difflugia corona*, for example, is apparently an omnivore preferring algae that extends its pseudopods in a manner extremely similar to that of *Chaos*. Pseudopod retraction, however, is accomplished much more rapidly by apparent contraction of a system of highly birefringent fibrils that seem almost to form by "crystallizing out of" the cytoplasm in the region between a pseudopodal attachment point and the body inside the shell (W o h l m a n and A l l e n 1964).

The shortening of *Difflugia* pseudopodia seems to be an active contraction

Table 2

Summary of observed differences related to movement (From G r i f f i n 1964)

Property	C. carolinensis, C. illinoisensis, A. proteus	Pelomyxa palustris
1. Characteristic form in locomotion	Polypodial (alternate pseudopods), ectoplasmic ridges, wrinkled tail	Monopodial, cylindrical body
2. Initiation of movement	Many pseudopods, gradual decrease in number	Extrusion of hyaline fluid over surface, single pseudopod
3. Hyaline cap or layer	Forms at front	Forms at rear
4. Reversal of direction	Frequent, wave of reversal from pseudopod base to tip	Ectoplasmic barrier forms and hyaline material is extruded at old front
5. Large inclusions	Carried in tail	Carried at front
6. Ingestion of food	Anterior food cups, motile food	Algae pulled in at tail
7. Light sensitivity	In anterior fountain zone, rapid response	Relatively insensitive, slow response
8. A_t/A_s ratio	Greater than one	Apparently less than one at times
9. Behavior of naked cytoplasm	Continues streaming in fountain and loop patterns	Contraction of tail of broken organisms, no streaming naked cytoplasm

because considerable work is performed in transporting the shell, the fibrils shorten and lose their birefringence, and syneretic blebs form near the fibrils along the length of the pseudopod. At the ultrastructural level, the fibrils are seen as densely packed bundles of microfilaments ca. 50 Å in diameter.

Pseudopods of testaceans seem to be more highly organized than those of the giant free-living amoebae (i.e., Chaos, Pelomyxa). They tend to be more perfectly cylindrical in shape when formed, more highly birefringent (positive with respect to the axis), and more likely to engage in waving or "searching" movements that play an apparent role in feeding behavior. Testaceans behave as though movement were under intricate central control; however, nothing is known about the control mechanism.

Helizoans have still more highly organized pseudopodia called axopodia. Each has a birefringent skeletal element, the axoneme, consisting of a double interlocking spiral array of microtubules (K i t c h i n g 1964, T i l n e y and P o r t e r 1966). Axopods can bend at the base, shorten or collapse totally. Dr. Christopher Watters from our laboratory carefully analyzed films of Actinosphaerium eichorni taken both from above and from the side, the later by means of the viewing chamber first described by D e l l i n g e r 1906. From his analysis, W a t t e r s 1966 was able to conclude that the "rowing motion" model of locomotion was illusory, and that shortening (and possibly bending) of leading axopods was responsible for locomotion (W a t t e r s 1966, 1968). Within individual axopods particles move in both directions, sometimes independently,

sometimes in coherent strings. T i l n e y and P o r t e r 1966 have attached considerable significance to the microtubules as possible motile organelles, but the studies of W a t t e r s indicate that particles may move at some distance (1 μ or more) from the axoneme. Some of these particle motions in the pseudopods can be classified as saltatory. Similar movements have been recorded and analyzed in the cortex by W a t t e r s 1966, who has designed a simple mathematical test for the conformity of particle motion data to that expected of brownian or saltatory motion.

Actinosphaerium catches food by the rapid collapse of axopodia to which ciliates of other food organisms have become attached. When the food organism has been drawn close to the cortical surface, more typically "amoeboid" pseudopods form a food cup in which the prey is rapidly engulfed.

Perhaps the most enigmatic type of pseudopodial movement is that found in the *Foraminifera* and *Radiolaria*. These marine protists form a typical "reticulopodial network" characterized by bidirectional streaming in all parts and a plasticity of form that is as intriguing as it is difficult to interpret (L e i d y 1879, J e p p s 1942). J a h n and R i n a l d i 1959 proposed an "active shearing" model based on the ubiquity of the bidirectional streaming. Some more detailed analyses of particle motions in *Allogromia* sp. (strain N.F. Lee) in our laboratory (A l l e n 1964) led to an alternate model based on multiple loci for generation of the motive force. The suspected loci are pseudopod tips and attachment points where new pseudopod branches are destined to form most frequently. While the motive force could be generated by some kind of active shearing (or "slidomatic") mechanism (J a h n and B o v e e 1964), it seems to be the rule that particles reversing direction at pseudopod tips and attachment points also lose or gain speed. In the smallest tips, particles advance toward the tip more rapidly than they return, suggesting that, if they are attached to some kind of fibrils (as their movement in coherent strings would suggest), the fibril must shorten where it bends and reverses.

Dr. S. M. McGee Russell and I are now engaged in a combined light and electron microscopic investigation aimed at studying the ultrastructure of reticulopodial networks, the previous histories of which are known from cinematographic records. There is still much uncertainty about reticulopodial ultrastructure because of difficulty in preserving fibrils strongly suspected of being present due to the birefringence of these pseudopodia. The fixation of reticulopodia has been done most successfully to date by A n g e l l 1967, who has managed to preserve tracts of microfilaments.

The details of movement exhibited by these five examples of giant amoeboid cells may be considered to argue against any general theory at the cellular level to explain all amoeboid movement processes, and diversity is even more apparent in the smaller free-living and parasitic amoebae, and in tissue cells of various organisms.

One must bear constantly in mind the fact that free-living amoeboid organisms occupy an enormous number of niches, and that the evolution of each has been influenced by different types of environment-specific selective pressures.

None of the models that have been erected to explain (at least in part) single cases of amoeboid movement cited in this paper are applicable to the other organisms discussed. With these differences in mind, it does not seem realistic at this time to divide all amoeboid movement processes into as few

as two categories as, for example, J a h n and B o v e e (1964) have done. At the cellular level, it would appear that many more mechanisms have evolved. At the ultrastructural level some amoeboid organisms have microtubules; other have microfilaments or still other linear elements. The roles of these structures and their molecular identity remain to be elucidated.

S u m m a r y

Amoeboid movement is often considered as a single phenomenon of wide occurrence. Recent studies by a number of investigators have begun to demonstrate some fundamental differences in the form, behavior, and ultrastructure of different types of pseudopods. Many of the differences have been demonstrated in film records of *Choas carolinensis, Pelomyxa palustris, Difflugia corona, Actinosphaerium eichorni,* and *Allogromia* sp. (strain N. F. Lee).

RÉSUMÉ

Le mouvenent ameboïde est souvent consideré comme un phenomène individuel d'une frequente occurence. Des études récentes des nombreux auteurs ont commencé à demontrer des différences fondamentales dans la forme, comportement et ultrastructure des différents types des pseudopodes. Un grand nombre de ces différences ont été demontrées sur pellicule. Les éspèces étudiées sont *Chaos carolinensis, Pelomyxa palustris, Difflugia corona, Actinosphaerium eichorni,* et *Allogromia* sp. (souche N. F. Lee).

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M. E. J. HOLWILL

Thermodynamic studies of the flagellar movement of some invertebrate spermatozoa

Les études thermodynamiques du mouvement du flagellum des spermatozoa des certains Invertebrés

In earlier papers (H o l w i l l and S i l v e s t e r 1965, 1967, H o l w i l l 1969) results have been reported to support the hypothesis that the beat frequency of a flagellum (hereinafter referred to as the frequency) is identical with, or at least proportional to, a rate constant of the first order chemical reaction which limits the frequency. The experimental evidence suggests that the rate-limiting reaction is the breakdown of a complex involving an enzyme and the energy-rich molecule adenosine triphosphate (ATP) which appears to be directly involved in the provision of energy for flagellar activity (e.g. B i s h o p 1962, B r o k a w 1962).

Certain flagella can be treated with solutions containing alcohol in such a way that they are rendered immotile, but can be made to beat again following the addition of solutions containing ATP (e.g. H o f f m a n n - B e r l i n g 1955, B r o k a w 1961, 1967). For some organisms the extraction procedure produces 'models' in which all the features of wave initiation and propagation are apparently preserved (B r o k a w 1962, 1963, G i b b o n s 1965, W i n i c u r 1968). The frequency of the models can be controlled by adjusting the ATP concentration of the medium surrounding the flagellum, so that this type of system is potentially suitable for studies to increase the information available concerning the thermodynamic aspects of flagellar activity.

The tails of the spermatozoa studied have the 9+2 fibrillar structure characteristic of eukaryotic flagella, having no ancillary features like those found in mammalian spermatozoa. This study is an extension of previous thermodynamic investigations into the behaviour of cilia and flagella from a variety of sources, many of which were protozoa. In view of the structural similarities between the sperm tails and protozoan flagella, results from the present study should be relevant to the movement of the latter type of organelle, and will be used in an attempt to further characterize the enzymic reactions which are responsible for flagellar motility.

Enzyme kinetics

Thermodynamics

According to a statistical treatment of reaction rates, the relation between a first order rate constant k and the absolute temperature T is given by:

$$k = \frac{\mathbf{k}T}{h} \exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right). \tag{1}$$

In this equation $\mathbf{k}$, h, and R are the Boltzmann, Planck and gas constants respectively while $\Delta G^{\ddagger}$ is the molar change in free energy that accompanies the activation of the chemical reaction. A similar expression is valid for equilibrium constants, but in this case the free energy change corresponds to the difference between the activation energies of the reaction in the forward and reverse directions.

The following equation relates the activation free energy with an activation entropy $\Delta S^{\ddagger}$ and enthalpy $\Delta H^{\ddagger}$:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}. \tag{2}$$

From equations (1) and (2) we have

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{\mathbf{k}}{h}\right) - \frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R} \tag{3}$$

so that an experimentally derived plot of $\ln \frac{k}{T}$ against $\frac{1}{T}$ should be linear and allow the calculation of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ from the slope and intercept of the line. [In earlier studies (H o l w i l l and S i l v e s t e r 1965, 1967, H o l w i l l 1969) the frequency was identified with a first order rate constant].

Briggs-Haldane kinetics

For an enzymic reaction of the type

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \overset{k_2}{\rightarrow} E + P \tag{4}$$
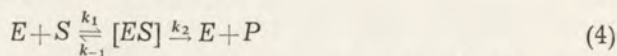
where E, S and P represent the enzyme, substrate and products, $[E S]$ is an enzyme-substrate complex and k_1, k_{-1}, k_2 are the rate constants for the individual reactions, B r i g g s and H a l d a n e (1925) derived the following general relation for the overall velocity, v, of the reaction:

$$v = \frac{k_2 \overline{K} [E_0] [S]}{1 + \overline{K}[S]}. \tag{5}$$

Here, $\overline{K} = \dfrac{k_1}{k_{-1} + k_2}$, $[E_0]$ is the total concentration of enzyme and $[S]$ is the substrate concentration.

For glycerinated flagella

$$v = \alpha f \tag{6}$$

(B r o k a w 1967, B r o k a w and H o l w i l l 1967) where f is the frequency and α is a constant. At concentrations sufficiently high that $\overline{K}[S] \gg 1$ we have, from equations (5) and (6),

$$\alpha f_{max} = k_2[E_0] \qquad (7)$$

where f_{max} is the frequency to which the flagellum tends as the substrate concentration is increased.

From equations (5), (6) and (7)

$$\frac{1}{f} = \frac{1}{f_{max}} + \frac{1}{f_{max}\overline{K}[S]} \qquad (8)$$

so that a graph of $\frac{1}{f}$ against $\frac{1}{[S]}$ should be linear and allow the calculation

of both f_{max} and $\overline{K}$ from the intercept and slope.

This treatment is analagous to L i n e w e a v e r and B u r k e's (1934) analysis of the Michaelis-Menten equation.

Material and methods

Spermatozoa from the sea-urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus*, the annelid *Chaetopterus vario pedatus* and the tunicate *Ciona intestinalis* were obtained and prepared by the methods described by B r o k a w (1965, 1966, 1967). Spermatozoa from the starfish *Pisaster brevispinus* and the mollusc *Megathura crenulata* were obtained by dissection and diluted with fresh sea-water for observation. Dr. R. L. Miller kindly prepared fresh suspensions of spermatozoa from the hydroid *Tubularia*. Frequency measurements were made stroboscopically, the organisms being viewed under conditions of dark field illumination.

The specimen temperature was controlled by passing heated or cooled water (containing ethylene glycol to prevent freezing at low temperatures) through the specially modified temperature stage. Measurement of the temperature was achieved by using a copper-constantan thermocouple, one junction of which was inserted in a hole bored through the centre of a microscope slide; a potentiometer arrangement allowed a null-point detection method to be used and suitable resistances were chosen to permit the temperature to be read directly from the dial of a helical potentiometer (H o l w i l l and S i l v e s t e r 1967). The calibration technique employed (H o l w i l l and S i l v e s t e r, loc. cit.) introduces a maximum error of $0.3°C$ in temperature over the range of the experiments. The scale could be read to within $\pm 0.1°C$.

Observations

Effects of temperature on frequency

The frequencies of intact spermatozoa from all the invertebrates which are listed under Material and Methods and of reactivated glycerinated spermatozoa from the sea-urchins were measured at a variety of temperatures between 5°

and 30°. For the glycerinated spermatozoa observations were made over a range of ATP concentrations from 10^{-4} to 10^{-3} M. For each frequency determination twenty spermatozoa were selected at random and an average frequency obtained; at a given temperature the frequency of the population was constant to within 10⁰/₀ of the average value. Since the frequency is known to vary with changing viscosity all frequencies were corrected for the viscosity change which occurs with changing temperature using the results of B r o k a w and H o l w i l l (unpublished work). The corrected frequencies are those expected in a medium of viscosity one centipoise.

For·all the intact organisms examined the graphs of ln f/T against $1/T$ were found to be essentially linear in the range 5—21°C. Above this temperature no correlation appears to exist between the parameters. A typical graph, that obtained from observations of *Ciona* sperm, is shown in Figure 1. From
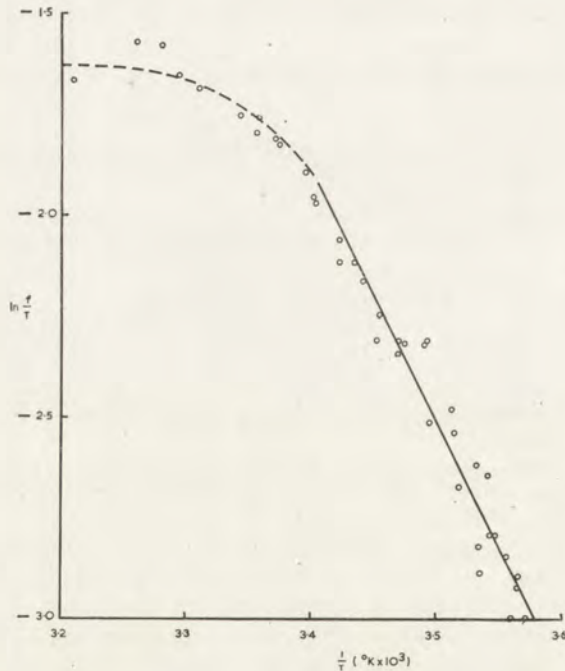

Fig. 1. Relation between frequency (f) and absolute temperature (T) for living spermatozoa of the tunicate *Ciona intestinalis*

each graph, average values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the quoted temperature range were calculated (Table 1). Because of the rapid deterioration of specimens above about 21°C, it was found impossible to investigate the movement on lowering the temperature.

.   The results of Table 1 are plotted on Figure 2 in which the straight line is the regression line of $\Delta H^{\ddagger}$ upon $\Delta S^{\ddagger}$ previously derived from observations on a large number of cilia and flagella (H o l w i l l and S i l v e s t e r 1967). Within the limits of experimental error all the points derived for the intact invertebrate spermatozoa examined here lie on the regression line.

Table 1

Values of the activation entropy ($\Delta S\ddagger$) and enthalpy ($\Delta H\ddagger$) for spermatozoa from a variety of marine organisms. These are mean values over the temperature range 5—21°C

Organism from which spermatozoa were derived	$\Delta H\ddagger$ (kcal/mole)	$\Delta S\ddagger$ (e.u.)
Lytechinus pictus (LL)	10.5 ± 0.3	-15.4 ± 0.5
Strongylocentrotus purpuratus (SL)	6.7 ± 0.3	-28.3 ± 1.0
Pisaster brevispinus (PB)	8.2 ± 0.2	-23.3 ± 0.1
Chaetopterus variopedatus (CV)	9.1 ± 1.2	-20.7 ± 1.0
Megathura crenulata (MC)	9.4 ± 0.4	-19.5 ± 0.1
Ciona intestinales (CI)	11.3 ± 0.3	-12.4 ± 0.3
Tubularia sp. (T)	10.8 ± 0.5	-14.3 ± 0.5

Letters in parentheses are the key to abbreviations used in Fig. 2.
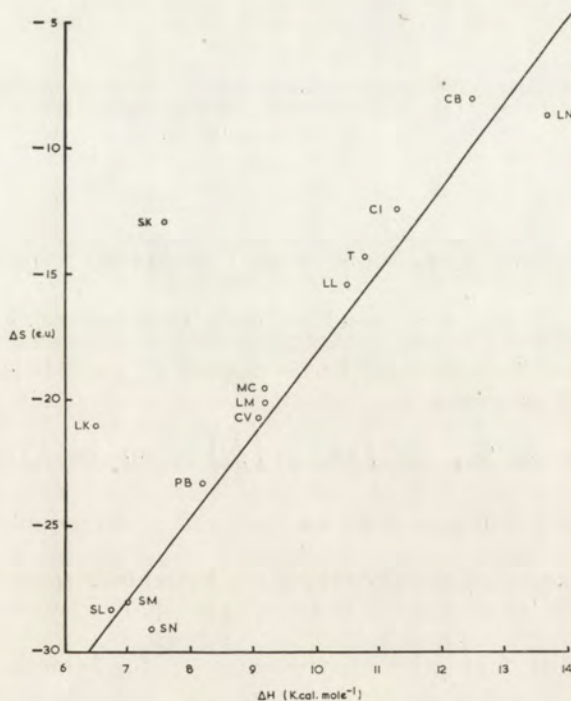
Fig. 2. Variation of the change in entropy (ΔS) with the change in enthalpy (ΔH) derived from the temperature dependence of various parameters. CB corresponds to the activation enthalpy and entropy for the ATP-myosin system. The key to the other abbreviations is given in Tables 1 and 2. The straight line is the regression line derived from the activation parameters calculated from observations of several cilia and flagella (H o l w i l l and S i l v e s t e r 1967)

Figure 3 shows the variation of the reciprocal of the frequency with the inverse ATP concentration for glycerinated models of *Strongylocentrotus* spermatozoa at several different temperatures. (Similar graphs were obtained
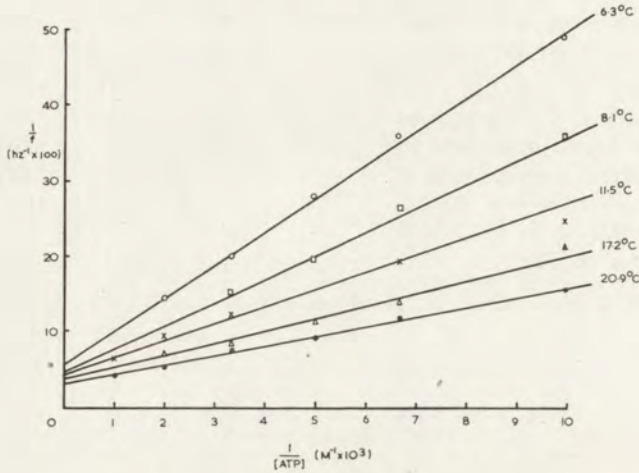

Fig. 3. Relation between frequency (*f*) and ATP concentration ([ATP]) at several different temperatures for glycerinated spermatozoa from the sea-urchin *S. purpuratus*

for glycerol extracted spermatozoa from *Lytechinus*). A linear relationship between $\frac{1}{f}$ and $\frac{1}{[ATP]}$ was found at each temperature, as predicted by equation (8). From the regression lines values of f_{max} and K (equation 8) were obtained at each temperature.

The variations of $\ln \left(\frac{f_{max}}{T} \right)$ and $\ln \left(\frac{f}{T} \right)$ at various ATP concentrations with $\frac{1}{T}$ are shown in Figure 4 for spermatozoa of *Strongylocentrotus* (again, graphs of a similar type were obtained for *Lytechinus* spermatozoa). All the plots appear to be linear and allow the calculation of activation entropies and enthalpies (Table 2). $\ln \left(\frac{\overline{K}}{T} \right)$ is plotted against $\frac{1}{T}$ for *Strongylocentrotus* spermatozoa in Figure 5. This curve, and the corresponding one for *Lytechinus* sperm are both linear over the range of temperatures in which studies were made, thus permitting the calculation of activation parameters (see Table 2) whose significance will be discussed later. In order to make comparisons with other results all the thermodynamic data calculated here are plotted on Figure 2.
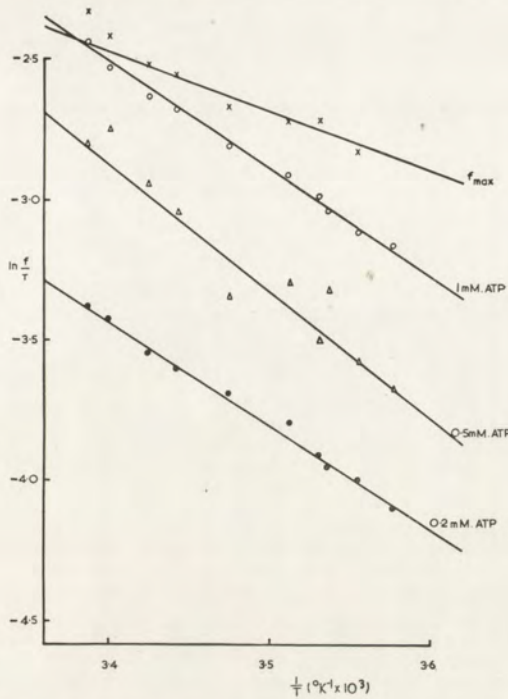
Fig. 4. Variation of frequency (f) with absolute temperature (T) at various ATP concentrations ([ATP]) for glycerinated spermatozoa from the sea-urchin *S. purpuratus*. The variation with temperature is also shown for the frequency (f_{max}) which is approached as the ATP concentration is increased

Table 2

Values for the change in entropy (ΔS) and enthalpy (ΔH) derived from observations on sea-urchin spermatozoa

Measured parameter	*Lytechinus pictus* sperm (L)		*Strongylocentrotus purpuratus* sperm (S)	
	ΔH (Kcal/mole)	ΔS (entropy units)	ΔH (Kcal/mole)	ΔS (entropy units)
Frequency, live sperm (L)	10.5 ± 0.3	-15.4 ± 0.5	6.7 ± 0.3	-28.3 ± 1.0
f_{max} sperm models (M)	9.2 ± 0.3	-20.1 ± 0.7	7.0 ± 0.8	-28.0 ± 1.7
Frequency at low ATP (N) cencentration, sperm models	13.7 ± 0.5	-8.7 ± 0.5	7.4 ± 0.5	-29.1 ± 2.0
$\bar{K}$ sperm models (K)	6.5 ± 0.5	-21.0 ± 2.0	7.6 ± 0.6	-12.9 ± 1.0

Letters in parentheses are the key to abbreviations used in Fig. 2: thus LM in Fig. 2 refers to activation parameters derived from measurements of f_{max} on *Lytechinus* sperm models.
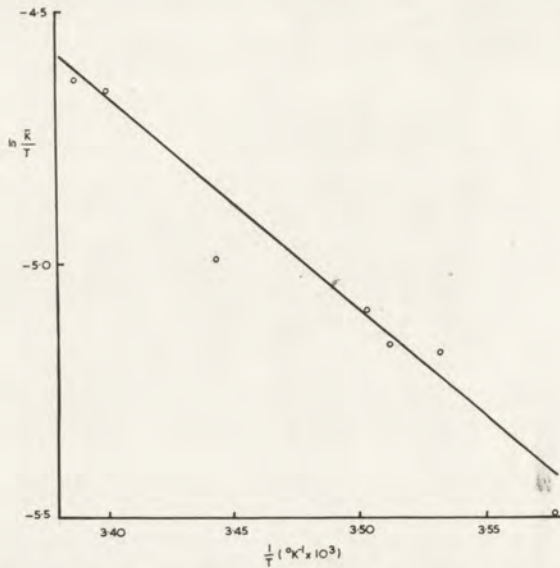
2*

Fig. 5. Variation of the constant $\overline{K}$ (see equation 2) with absolute temperature (T) for glycerinated spermatozoa from the sea-urchin *S. purpuratus*

Discussion

The curvature at the higher temperatures shown by the graphs of which Figure 1 is an example provide further confirmation that the frequencies in this temperature range are lower than those required to give a consistent linear relationship between $\ln \dfrac{f}{T}$ and $\dfrac{1}{T}$ (H o l w i l l 1969). Enzymes respond to changes in temperature in a manner very similar to the curve of Figure 1, with the frequency replaced by the reaction velocity. In this case, the curvature is attributed to enzyme denaturation as the temperature is increased. For the spermatozoa of all the marine organisms examined in this study, the curvature (and presumably the denaturation of the enzyme involved in the chemical reaction which provides energy for flagellar activity) occurs at a temperature lower than that for other organisms (H o l w i l l and S i l v e s t e r 1967). There are at least two conditions which could account for this observation. First, there could be structural variations in the enzymes from one organism to another so that to produce denaturation a variable number or type of chemical bond needs to be broken; the energy required for denaturation (and hence the effects of temperature) will change from one species to another. Second, the enzymes could be identical in all organisms but exposed to different environmental conditions (e.g. pH, dielectric constant, etc.) which are such as to favour denaturation at lower temperatures in the spermatozoa of marine organisms than in others. The effect may be the result of the fact that marine organisms normally experience lower environmental temperatures (in the region of 16°C) than many of the organisms previously examined by H o l w i l l and S i l v e s-

t e r (1967). Certain parasitic protozoa, for instance, are normally exposed to temperatures of the order of 37°C.

The entropies and enthalpies calculated from the studies of the effect of temperature on frequency for all the organisms examined in this study conform to the linear pattern of activation parameters obtained earlier for a number of ciliates and flagellates (see Figure 2 and H o l w i l l and S i l - v e s t e r 1967), thus suggesting that the chemical reaction which limits the frequency of sperm tails is similar to that in other flagellates. It is interesting to note that, within the limits of experimental error, the thermodynamic para- meters characteristic of the rate limiting step in live spermatozoa of the two sea urchins are the same as those in the respective glycerinated models. This supports the suggestion that the reactions in vitro are identical with those in vivo, but further work on a variety of glycerinated flagellates is needed to test this conclusion.

According to equation (7), since the total concentration of enzyme (E_0) is constant, f_{max} is proportional to k_2 so that the temperature dependence of f_{max} essentially represents that of k_2. Since k_2 is the rate constant characteristic of the breakdown of an enzyme-substrate complex (equation 4) the activation parameters that would be derived from a plot of $\ln\left(\dfrac{k_2}{T}\right)$ against $\dfrac{1}{T}$ are those corresponding to this breakdown. Experimentally, it is only possible at present to plot a graph of $\ln\left(\dfrac{f_{max}}{T}\right)$ against $\dfrac{1}{T}$ (Fig. 4) so it is of interest to examine how the thermodynamic quantities derived from this graph are related to those of complex breakdown. The activation enthalpy, being calculated from the slope of the graph, will have a value that is independent of the magnitude of the constant $E_{0/\alpha}$ in equation (7), and hence represents that of the complex breakdown. The activation entropy, on the other hand, is evaluated from the intercept of the graph so that the extrapolation from the experimentally derived entropy to that characteristic of the breakdown of the complex depends on the value of $E_{0/\alpha}$ If each enzyme molecule is used only once per beat (the available experimental evidence does not contradict this (B r o k a w 1967)), then the constant $E_{0/\alpha}$ is unity. In the event that each enzyme molecule is used more or less than once per beat, the linearity of Figure 2 indicates that the constant $E_{0/\alpha}$ is the same for all flagella so far examined and is of such a value as to allow the point corresponding to the breakdown of an ATP- myosin complex to fall close to the line. The activation parameters derived from observations of glycerinated spermatoza are evidently closely related, if not identical, with those of the breakdown of an enzyme-substrate complex. The close correspondence of the entropies and enthalpies obtained from the live spermatoza and the f_{max} of glycerinated spermatozoa supports the earlier conclusion (H o l w i l l and S i l v e s t e r 1967) that the rate-limiting step within a flagellum is the breakdown of an ATP-ATPase complex.

It is of interest to discuss the activation parameters derived for glycerinated spermatozoa in terms of the enzymic reaction (equation 4) used to derive the Briggs-Haldane equation (equation 5). Equation (4) may not, however, be truly representative of the reactions occurring within the flagellum; it is possible that there are intermediate reactions between those specified in equation (4).

The intermediate reactions would not necessarily alter the form of the Briggs-Haldane equation, but would certainly alter the significance of the constant $\overline{K}$ and possibly also k_2 (see, for example, L a i d l e r 1958).

For sufficiently low substrate concentrations equations (5) and (6) reduce to

$$v = \alpha f = k_2 \overline{K}[E_0][S]. \tag{9}$$

Since $[E_0]$ and α are constants the variation of f at a single low substrate concentration reflects the variation of the complex constant $k_2\overline{K}$. The linear relationship that exists between ln (f/T) and $\frac{1}{T}$ at concentrations of ATP in the range 10^{-4}—10^{-3} M suggests that $k_2\overline{K}$ is a rate constant, an equilibrium constant or the product of two such constants since it is only under these special conditions that linearity is to be expected. Since $\overline{K} = \frac{k_1}{k_{-1}+k_2}$ it is clear that if either of the inequalities $k_{-1} \gg k_2$ or $k_{-1} \ll k_2$ is valid, then two of the special conditions noted above are possible. If $k_{-1} \ll k_2$ then $\overline{K} = \frac{k_1}{k_2}$ and $k_2\overline{K}$ is the rate constant k_1. When $k_{-1} \gg k_2$, K is the equilibrium constant $\left(\frac{k_1}{k_{-1}}\right)$.

The energy relationships for the two cases are different (see Figure 6) so that it is important to establish which applies to the flagellum. The distinction cannot be made on the basis of the present results, so that further experiments
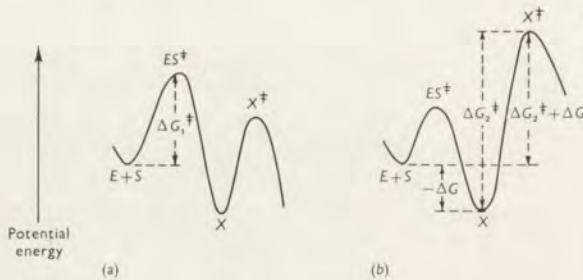

Fig. 6. Schematic energy diagrams for enzyme reactions which may be represented by equation 1: (a) when $k_2 \gg k_{-1}$ and (b) when $k_2 \ll k_{-1}$, $[ES]^{\ddagger}$, X and $X^{\ddagger}$ are three forms of the enzyme complex, the superfix‡ indicating the activated state

designed to investigate more fully the type of reaction responsible for flagellar movement are necessary before the real nature of the reaction can be resolved.

To be consistent with the results, if simple Briggs-Haldane kinetics apply, then k_{-1} should be larger than k_2 leading to the conclusion that the limiting chemical reaction of flagella is the breakdown of an enzyme-substrate complex.

It is interesting to notice that in addition to satisfying equation (8), the square of the frequency of glycerinated spermatozoa is linearly related to the ATP concentration over the range employed in the present experiments (Fig. 7).
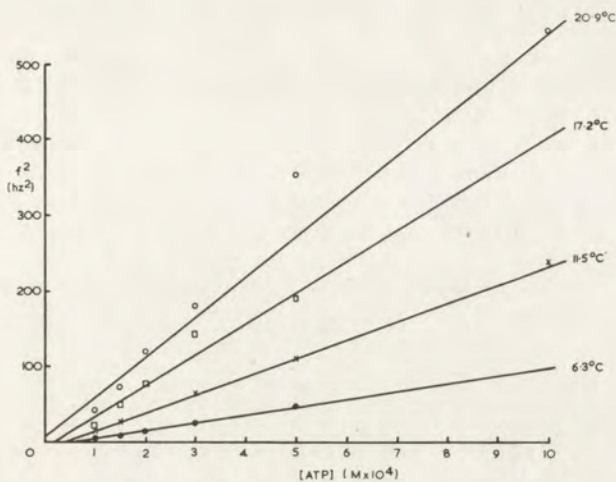
Fig. 7. A further relation between frequency (f) and ATP concentration ([ATP]) at several different temperatures for glycerinated spermatozoa from the sea-urchin *S. purpuratus.* (See also Fig. 3)

This type of behaviour is predicted by C. A. M i l e s (personal communication) who has studied the tension dependence of the enzymic reaction for simple contractile and sliding filament models of the machinery responsible for moving the flagellum. A detailed account of this work is in preparation.

Acknowledgements

S u m m a r y

From a study of the thermal dependence of flagellar activity in a variety of invertebrate spermatozoa values for activation enthalpies and entropies are calculated that pertain to a chemical reaction within the flagellum. This reaction is involved in the conversion of chemical to mechanical energy in the flagellum. The activation parameters for glycerol-extracted spermatozoa are the same as those for the corresponding live spermatozoa, thus suggesting that the mechanochemical reactions are the same in vitro as in vivo.

RÉSUMÉ

Dans un étude de la dépendance de l'activité du flagellum de la temperature chez un nombre des spermatozoa des Invertebrés les valeurs des entalpies de l'activation et de l'entropie calculées indiquent une réaction chimique a l'interieur du flagellum. Cette réaction est liée a la conversion de l'energie chimique en mécanique dans le flagellum. Les paramètres de l'activation pour les spermatozoa extraits avec du glycerol sont les mêmes que ceux correspondant aux spermatozoa vivants indicat de cette façon que les réactions mécanochimiques sont les mêmes in vivo ainsi que in vitro.

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L. N. SERAVIN

Left and right spiralling round the long body axis in ciliate protozoa

Лево- и правовращение ресничных инфузорий вокруг продольной
оси тела

All the known ciliates — when swimming in water — rotate round their long body axis (Bullington 1925, 1940). Some of them rotate right (clockwise direction), the others — left (anticlockwisely), when observed along the body surface from the posterior towards the anterior end. Jennings 1901 postulated that three factors determine the rotation of *Paramecium* around its long body axis and the spiral way of their movement: 1. assymmetry of the body, 2. oblique position of peristome, 3. oblique (to the long body axis) direction of ciliary beat. Bullington 1925 investigated precisely the movement of 164 forms of ciliate protozoa. He stated that 102 species rotate left round the long body axis, and 62 species — right. Besides, he succeded to demonstrate that the direction of rotation is independent of the size and form of the body.

In a number of citiate species (among them in some *Paramecium* species) on their anterior body end, an oblique peristomal groove exists which gives the form of a screw to the ciliate body. Hydrodynamic factors could involve a right rotation in those ciliates. What is this movement indeed? The presence of oblique peristome has been stated by Bullington in 67 species out of 164 investigated. In 48 species, rotation was left i.e. anticlockwise, being right in 19 species only. Analysing the data obtained Bullington came to the conclusion that the rotation of the ciliate body is involved by an effective stroke of cilia performed under an angle to the long body axis. The shape of the body exerts no influence in this phenomenon. This point of view was supported by Párducz 1962, 1967. In his opinion, every ciliate has two regulatory mechanisms, one of them is determining the left rotation, the second — the right one around the long body axis.

In the recent time however some interesting publications appeared supplying new evidences that — at least in paramecium — the body shape may influence the direction of spiralling at some definite conditions (Kuźnicki and Sikora 1966, Grębecki, Kuźnicki and Mikołajczyk 1967 a, b). The principal conclusions of those studies are as follows.

1. In the *Paramecium* species of the group "aurelia" (*P. caudatum, P. aurelia, P. multimicronucleatum*) only the left spiralling (LS) occurs in natural conditions.

2. The right spiralling (RS) arises in an unspecific way under the action of different factors upon cilia (rise of the medium viscosity, Ni-ions, homologeous antiserum, low temperature) i.e. at a sufficiently high fall of the movement speed.

3. The transition from LS to RS occurs after the action of mechanical (hydrodynamic) factors because the body of paramecium has the form of a right spiralled screw owing to the oblique peristome. In conditions which interfere with the ciliate movement, a turbine effect arises associated with the screw-shaped body of paramecium. So RS becomes more effective hydrodynamically.

4. The Ca ions fail to influence considerably the transition from LS to RS, at any rate there is no interdependence observed in the direction of ciliary reversal i.e. this transition is not connected with the changes in the sensibility of the cell.

5. The transition from LS to RL is evoked by purely hydrodynamic factors. As a result, reversal of the ciliary beat direction takes place promoting the right spiralling of the ciliate body. Consequently, the above results may be considered as supporting the hydrodynamic theory of ciliary beat in *Protozoa*.

Taking into account the theoretical importance of the above results, we found possible and necessary to follow once more the question of the influence of the body shape upon spiralling in ciliates. This seems to be the more of importance since the investigators who accept any of those two controversive theories base their point of view on indirect evidences.

The influence of Ni ions and of the rise of the medium viscosity upon the inversion of spiralling direction in fragments of paramecia

Ni ions or the rised medium viscosity easily influence appearing of the right spiralling forms in the "aurelia" group of paramecia (A l v e r d e s 1922, L u d w i g und S c h l i c k s u p p 1951, G r ę b e c k i et al. 1967 a, b). Owing to this fact, it is rather easy to ascertain experimentally what role is played in the inversion of the spiralling direction by the peristomal groove which runs in a screw-like course along the anterior body part. For this it was necessary to cut the ciliate body into two parts in the region of the oral aperture. The whole peristomal groove proves to be in the anterior fragment, the posterior one being deprived of it. If the "turbine effect" i.e. hydrodynamic factor played an essential role in appearing of right spiralling, it should be expected that — after the rise of viscosity or action of Ni ions, the posterior fragment would keep LS whereas the bent screw-like anterior fragment would begin the right spiralling round its long body axis, similarly as the intact individuals.

Control experiments were carried out. *P. caudatum* was cultivated — like in all the other cases — on lettuce medium according to the generally accepted method (S o n n e b o r n 1950). The geotactic reaction was applied (D r y l 1961) followed by rinsing in the L o s i n a - L o s i n s k y 1931 medium in which the $CaCl_2$ content was raised to 1 mM). The bisection of paramecia into two fragments in the zone of the oral aperture was executed by means of a glass needle. 63 posterior and 56 anterior fragments were used in experiments. 10 min. after isolation, the fragments showing LS only were placed in solution of $NiSO_4$ (0.001—0.0005%) or in carboxymethyl cellulose (0.5%). The results indicated that right spiralling occurred in the anterior as well as in the posterior frag-

ments in both cases. Consequently the inversion of spiralling does not depend on the body shape of paramecium and is not determined by the hydrodynamic fractors.

Influence of Ni ions upon the inversion of spiralling in the intact ciliates

It is considered that Ni ions induce the change of LS for RS in *Paramecium* (P á r d u c z 1962, G r ę b e c k i et al. 1967 b). Experiments were carried out for proving this fact. For taking adventage of the geotactic reaction, the ciliates were rinsed twice in one of the following solutions: 0.03 M K-Na-phosphate buffer (pH 7.0), 1 mM $CaCl_2$ solution and bidistilled water. Paramecia rinsed in this way were placed in $NiSO_4$ solutions in bidistilled water.

In the ciliates kept previously in the solution of $CaCl_2$, under the influence of Ni ions (0.0002—0.005% $NiSO_4$), in the course of gradual immobilization, a steady RS occurs. Another picture is observed when the preliminary rinsing was carried out in a medium without the ions or in the phosphate buffer containing the univalent ions only. In this case, in *P. caudatum* placed in $NiSO_4$ solution, the permanent RS is never observed but only an alternate change of LS into RS and inversely (LS ⇄ RS). This change takes place at all the stages of a gradual immobilization and fails to depend on the speed of the ciliate movement. It may occur also in the individuals with no progressive movement which are capable of rotation at one place only.

The dependence of the Ni ions action upon the inversion of spiralling in the presence of K and Ca ions in the medium is especially clearly seen in the two following series of experiments. Their results are shown in the Tables 1 and 2.

If in the medium only the Ca ions are present, then $NiSO_4$ evokes a permanent RS in paramecia. In the presence of K ions $NiSO_4$ evokes a periodic change LS ⇄ RS. The same occurs in the case when the solution contains no ions except for $NiSO_4$ and paramecia have been previously rinsed in bidistilled water.

If Ca and K ions are present in the medium simultaneously, the effect would depend on the ratio of those ions in the medium. If Ca^{++} dominate, a permanent RS arises. When K ions prevail, a periodic inversion LS ⇄ RS is observed. Similar results have been gained with *P. aurelia* and *P. woodruffi*.

Table 1

Dependence of inversion LS ⇄ RS in *Paramecium caudatum* on concentration of Ni, Ca and K ions in medium

% $NiSO_4$	Bidistilled water	$CaCl_2$ 2 mM	$CaCl_2$ 1 mM	$CaCl_2$ 0.5 mM	KCl 2 mM	KCl 1 mM	KCl 0.5 mM
0.005	LS ⇄ RS	—	RS	RS	LS ⇄ RS	LS ⇄ RS	LS ⇄ RS
0.001	LS ⇄ RS	RS	RS	RS	LS ⇄ RS	LS ⇄ RS	LS ⇄ RS
0.0005	LS ⇄ RS	RS	RS	RS	LS ⇄ RS	LS ⇄ RS	LS ⇄ RS
0	LS	LS	LS	LS	LS	LS	LS

Table 2

Influence of K and Ca ions ratio in medium upon inversion
LS ⇄ RS evoked by Ni ions in *Paramecium caudatum* (In
all cases the concentration of $NiSO_4$ — 0.0005%)

CaCl₂ / KCl	2 mM	1 mM	0.5 mM	0 mM
2 mM	RS, then LS	LS ⇄ RS	LS ⇄ RS	LS ⇄ RS
1 mM	RS	LS ⇄ RS then RS	LS ⇄ RS	LS ⇄ RS
0.5 mM	RS	RS	LS ⇄ RS then RS	LS ⇄ RS
0 mM	RS	RS	RS	LS ⇄ RS

Since in this case LS and RS are observed in the same individuals in the process of immobilization at the same low speeds of movement, the inversion LS ⇄ RS cannot be accounted for by the hydrodynamic factors (turbine effect). It is more adequate to postulate, following P á r d u c z 1962, 1967, that in *P. caudatum* two regulatory mechanisms exist: one of them defining the left spiralling, the other — the right one. The Ni ions provide conditions in which both mechanisms may act simultaneously which is reflected in their alternate periodical activity. In the presence of $NiSO_4$, the Ca ions supress the action of the mechanism which secures LS. The Ca^{++} effect may be suppressed by K^+.

The following experiments indicate that Ni ions evoke in ciliates a simultaneous action of two different mechanisms which define the direction of spiralling of the body.

P. calkinsi similarly as *P. caudatum* has a well developed peristomal groove which gives to the ciliate the form of a right-round screw. However in the normal conditions, *P. calkinsi* — in contrast to *P. caudatum*, when swimming spirals right round its long body axis (B u l l i n g t o n 1925, 1930, W i c h t e r m a n 1953). Consequently if the postulation that the inversion of spiralling was determined by the body shape (hydrodynamic factors) was right — then in *P. calkinsi* in $NaSO_4$ solutions the RS should persist as more convenient (G r ę b e c k i et al. 1967 b). However the experiments performed proved that in the process of immobilization of those ciliates in solution of $NiSO_4$ (0:002—0.01%) the LS appears or — more precisely — a periodical alternation of RS ⇄ LS. In the other words, a simultaneous action of the different mechanisms which determine the direction of spiralling around the long body axis in paramecium is observed. Applying the Ni ions the same results may be gained in the experiments with other ciliates, not related to the genus *Paramecium* (Table 3). It should be remarked besides that:

Table 3

Influence of NiSO₄ (0.001—0.01%) on spiralling direction in different ciliates

Ciliate species	Spiralling direction in culture medium	Spiralling direction in presence of Ni ions
Lionotus fascicola	LS	LS ⇄ RS
Coleps hirtus	LS	LS ⇄ RS
Spirosotomum ambiguum	LS	LS ⇄ RS
Trachelius ovum	RS	RS ⇄ LS
Dileptus anser	RS	RS ⇄ LS
Carchesium polypinum	RS	RS ⇄ LS
Euplotes patella	RS	LS
Stylonychia mytilus	LS	LS
Urostyla grandis	LS	LS
Stentor coeruleus	RS	RS
Colpidium colpoda	RS	RS
Didinium nasutum	RS	RS

1. The simultaneous action may be evoked in the ciliates which are left-spiralling in the normal conditions (*Lionotus, Coleps, Spirostomum*) as well as in those which are right-spiralling (*Trachelius, Dileptus, Carchesium, Euplotes*).

2. The simultaneous action of the regulating mechanisms is observed in the forms in which the peristome is not oblique (*Coleps, Dileptus* etc.).

3. This phenomenon is rather difficult to be evoked at conditions of Ca ions in the medium in such ciliates as *Spirostomum*.

4. In some ciliates the inversion of spiralling cannot be evoked by means of Ni ions (Table 3).

After the evaluation of the results gained, a conclusion may be drawn that appearing of RS instead of LS and inversely does not depend on the body shape of the ciliate i.e. is not determined by hydrodynamic factors. This process, like any other biological phenomenon, depends of the ratio of Ca and K ions in the medium.

The influence of medium viscosity on the inversion of spiralling in ciliates

In the experiments of this series, the solutions of carboxymethyl cellulose (CMC) were used. In 0.5% solution of this substance, at the same speed of *P. caudatum* movement, not RS arises but a periodic alternation of LS ⇄ RS, however with domination of RS. Quite similar results were gained in the experiments with *P. aurelia* and *P. woodruffi*.

If the inversion of spiralling in paramecia evoked by the rise of viscosity was tetermined by the hydrodynamic factors (turbine effect), then it should be expected that in the right spiralling *P. calkinsi* only an intensified RS would appear. However the experiments carried out fail to prove this effect. In 0.5% solutions of CMC left spiralling arises in *P. calkinsi* changing for the right

one from time to time (RS ⇄ LS). In other words, the spiralling direction does not depend on the body shape of *Paramecium* and is not determined by the hydromechanical agents. Similar results have been obtained with some other ciliates (Table 4). Besides, it should be remarked as follows: 1. the inversion

Table 4

Influence of corboxymethyl cellulose (CMC) on direction of spiralling in different ciliates

Ciliate species	Concentration of CMC in medium, %	Direction of spiralling in culture medium	Direction of spiralling in solutions
Lionotus fascicola	0.125—0.25	LS	LS ⇄ RS
Coleps hirtus	0.135—0.25	LS	LS ⇄ RS
Spirostomum ambiguum	0.125	LS	LS ⇄ RS
Colpidium colpoda	0.25	RS	RS ⇄ LS
Stentor coeruleus	0.125—0.5	RS	RS
Dileptus anser	0.125—0.5	RS	RS

of spiralling may be evoked as well in the left-spiralling species (*Lionotus, Coleps, Spirostomum*), as in the right spiralling forms (*Colpidium*), 2. inversion may appear in those in which the peristomal groove is absent (*Coleps, Colpidium*), 3. inversion may be observed in those forms in which it is not induced by the Ni ions (*Colpidium*), 4. inversion may not be manifested in those species in which it is evoked by Ni ions (*Dileptus*).

In this way, the rise of medium viscosity evokes in ciliates not RS but, similarly as the Ni ions, corroboration of two different mechanisms which determine the direction of spiralling. This indicates that the rise of viscosity acts not only upon functioning of cilia but also, directly or indirectly, upon the regulatory of ciliates.

Inversion of spiralling direction of ciliates in natural conditions

The spiralling inversion in cilates swimming in natural conditions was investigated more or less in details in the representatives of three genera: *Paramecium, Frontonia* and *Nyctotherus*. It was shown by the studies of Bullington 1930 that the species of the genus *Paramecium*, belonging to the group "bursaria", are left-spiralling forms but may sometimes rotate right when swimming (*P. bursaria, P. polycarium, P. woodruffi*). An exception presents *P. calkinsi* in which a permanent RS is natural. Bullington found in *P. trichium* a permanent, never changing LS. However the subsequent study of Bragg 1934 demonstrated that *P. trichium* may spontaneously change LS for RS in the natural conditions.

A widely accepted meaning exists that in paramecia of the type "aurelia", exclusively LS is observed in natural conditions (Wichterman 1953, Grębecki et al. 1967 b). However Bullington 1930 established that in *P. aurelia* RS may also be observed although LS predominates. Rosenberg 1937 reported his observation of the inversion of spiralling direction in

P. caudatum and *P. multimicronucleatum.* L u d w i g und S c h l i c k s u p p 1951 presented evidences proving that in *P. caudatum* RS appears as frequently as LS. We succeeded to observe the inversion of spiralling in very well fed individuals of *P. caudatum* and *P. aurelia* sampled from a ring which was formed by the ciliate in a culture after feeding with bacteria.

R o s e n b e r g 1937 postulated that in all the representatives of the genus *Nyctotherus* a spontaneous change LS ⇄ RS occurs in movement. At any rate, he observed the inversion of spiralling in the natural conditions in *N. cordiformis, N. ovalis, N. hylae* and *N. velox.* P á r d u c z 1967 supported those findings for *N. cordiformis.*

B u l l i n g t o n 1939 investigated the pecularities of movement in eight ciliate species of the genus *Frontonia.* Two of them proved to be left-spiralling (*F. atra* and *F. acuminata*). The representatives of the two others were right-spiralling (*F. vernalis* and *F. ocularis*). The remaining 4 species alter periodically LS ⇄ RS in the normal conditions. Those are: *F. leucas, F. vesiculosa, F. marina* and *F. schafferi.* However no one of those four species spirals evenly in both directions, one of them is predominating. So, in the first 3 species, the left spiralling occur most frequently, and in *F. schafferi* — the right one.

Those scarce data permit to draw conclusion that, as a rule, two different mechanisms exist in ciliates which determine the direction of spiralling. In a number of forms, both mechanisms are functioning by turn which involves a frequent inversion. In the other species, the work of one mechanism is supressed and then — one-side spiralling prevails.

By a number of agents (Ni ions, rise of viscosity etc.) the action of both mechanisms may be evoked experimentally. Then a periodical alternation arises LS ⇄ RS or RS ⇄ LS. Possibly some species of ciliates have lost one of those mechanisms and then the inversion of spiralling cannot be evoked even experimentally.

Peculiarities of the water streams on the body surface of an arrested *Paramecium* (preliminary results)

For observing the course of water evoked by cilia at the very surface of *Paramecium caudatum* body, suspension of ink or of carmin was added to the culture medium. Then a drop of this culture liquid was placed on a slide, covered with a glass after having secured the margin of the drop with a thread. The slide was placed on the microscope stage. A thin metal rod was adjusted at a 45° angle to a tripod with a microscrew. The tip of the metal rod was touching the surface of the cover glass. By turning the microscrew a regulated pressure could be produced by rod, which resulted in controlling the pressure upon the ciliates in the drop.

In the very strongly pressed individuals, on their free margines, a coordinated work of cilia persisted. Along the surfaces, two uninterrupted streams of the culture medium produced by cilia were observed. Big suspended particles which are present in the streams, move and rotate around their axis. Small particles perform, besides this, circulating movements. In this way every cilium, when acting, gives to the particles of the medium (as well as to the particles suspended in it) not a straight-lined but a rotatory movement. The water streams produced along the ciliate body consist of a complex of small whirls moving from the anterior toward the posterior body part.

If the ciliate is not too much pressed, but sufficiently for not being able to rotate around its long axis and to move very slowly under the cover glass — cilia work normally. Then on the free body surface, two very strong water streams are formed. The suspended particles move in those streams not along straight lines but follow an extended spiral. Both streams rotate round their long axis in the same direction: in some individuals to the right in the others — leftwards.

It the pressure of the cover glass is quickly reduced, the ciliates which produced the streams spiralling right — begin to move spiralling left round their long body axis. Inversely, the individuals of the left streams, move spiralling right (pressure may induce the right spiralling).

The existence of whirl streams may be observed not only in the pressed ciliates, they are also manifested in free individuals which stopped for feeding at the bottom of microaquarium in the area of accumulation of bacteria. Similar streams may be observed in the other ciliate species e.g. in *Frontonia leucas* and *Bursaria truncatella*. Consequently they are not a peculiar feature of paramecia only.

Discussion and the preliminary hypothesis

The experiments with the influence of Ni ions and with raised medium viscosity upon the fragments of *Paramecium caudatum*, upon the intact individuals of *P. caudatum* and *P. calkinsi*, as well as upon the other ciliate species (including those without the peristomal groove), demonstrate that the direction of spiralling of the ciliate body and the inversion of this direction fail to depend on the shape of the ciliate body, and are not determined by the hydrodynamic factors but by some other agents.

Evidently in all, or in many, ciliated protozoa two regulatory mechanisms exist which secure the spiralling of the ciliate around its long body axis. In the natural conditions, the action of one of them dominates although that of the other kind may also be observed. External factors, including the Ni ions and rise of the medium viscosity, may evoke engaging of both mechanisms, resulting in a periodical change LS ⇄ RS.

In our opinion, most striking is the fact of independence of spiralling direction of the individual moving in liquid from its body shape. However the ciliates supply a number of cases of deviation of the hydrodynamic rules. So many of those ciliates have a poorly streamline body shape. Besides the spherical ciliated protozoa, some other types should be mentioned in this respect: *Stentor* or *Paradileptus* with an enlarged and flatly cut anterior end, ciliates of the *Teuthophrys* type with their anterior end in the form of a concave cup which in *Bursaria* is even sack-shaped. From the hydrodynamic point of view the body shape of those ciliates interferes with the movement of water. However the above ciliates are able to swim effectively.

The ciliated protozoa swim spiralling around their long body axis. In this case, a slightest hydrodynamic resistance should be met by the spherical, cylindrical or another similar forms. However among *Protozoa* flattened forms occur not rarely. So some *Hypotricha* have a plate-like shape which involves conditions interfering with spiralling. Nevertheless those ciliates swim spiralling around their long body axis.

In this way, the facts gained at present time speak in favour of the hypothesis that the body shape plays no essential role in the movement of ciliates.

In the study of hydrodynamics of a moving object it is important to know what forces, of inertion or of viscosity, predominate in the stream of liquid on the surface of the object. The ratio of those forces is characterized by the number of Reynholds (Re). In the classic hydrodynamic, the regularities of the body movement in the liquids of a high value of Re, when the inertion forces dominate, have been investigated extensively. Much less known are those regularities in the case of the low Re values — when the viscosity forces dominate. For the moving ciliated protozoa very low values of Re — below 1 (K o k s h a i s k i j 1967) are characteristic. The resistance of medium to the body of a high Re value rises proportionally to the square of the movement velocity (V^2) of this body. In this case, any deviation of the form of a highly streamline body evokes an abrupt rise of the frontal resistance. For the moving bodies at $Re \leqslant 1$, the resistance of medium rises proportionally to the movement velocity of the body (V). In these conditions, the fluctuations of the body shape are evidently less essential.

However it is difficult to explain by this factor only the movement of individuals with a fairly streamline body; possibly another causes exist as well. One of them has not been discussed in the scientific literature as yet.

In animals like fishes, mammals, usually only one propelling agent exists, being localized in one definite place (tail, paws etc.). This agent secures the forward movement of the animal. The medium resists to it by its passive flow. Its resistance, despite the other factors, depends on the body shape. In the majority of ciliates many propellent factors exist being distributed over the whole body surface. Cilia working metachronically not only secure the progressive movement but actively — in a definite way — produce organized and directed streams round the surface of the body. The surrounding liquid meets those streams and not the passive surface of the moving body. A compulsory active flow of liquid over the ciliate body occurs, and consequently any resistance is being determined not by the shape of the ciliate body but by a system of streams which arise as a result of the work of cilia. Evidently if not the whole body surface is covered with cilia, streams arise which secure the flow on the "naked" areas.

The observation of the movement of small suspended particles round the surface of a compulsory arrested paramecia indicate that the water streams produced by the movement of its cilia have a whirly nature. Therefore the rotating particles move in the stream following a spiral curve. The common liquid stream produced by paramecia, rotates around paramecium as well in the direction inverse to the spiralling of the ciliate body. The stream in which the liquid circulation and rotation of particles is observed, is called in hydrodynamic terms a whirl stream. Observations indicate that not only paramecia but also other ciliates swim producing similar streams. We put forward a working hypothesis which explains some peculiarities of the movement and of the hydrodynamics of ciliated protozoa. It may be conventionally called the hypothesis of whirl stream. The main propositions of this hypothesis are as follows:

1. The movement of ciliates occurs owing to a system of whirl streams produced by the ciliary system of the ciliate. In result, the body of the ciliate

spirals around the long body axis in the direction inverse to the rotation of the common whirl stream.

2. The movement and spiralling of ciliates depend not so much on the body shape as on the system of whirls which produce a compulsory flow of the surrounding liquid over the ciliate body.

3. The ciliate is capable to inverse the spiralling of its body and the direction of its movement by regulating the direction of spiralling and the movement of the common whirl streams.

4. Inhibition or stimulation of the action of different areas of the ciliary covering, enables the ciliate to modify the form of the common whirl stream which, in result, changes the character and form of its movement.

Evidently this work hypothesis needs to be exactly revised experimentally.

Summary

Experiments were performed on the action of Ni ions and of rise of medium viscosity in fragments of *Paramecium caudatum*, on unimpaired *P. caudatum* and *P. calkinsi*, as well as on other ciliate species including those with no peristomal groove. The results indicate that the direction of spiralling of the ciliate around its long body axis and inversion of this spiralling depend not on the form of the ciliate body and are not determined by hydrodynamic factors but by some other agents. Presumably in all (or in many) ciliated protozoa, two regulatory mechanisms exist which secure spiralling of the ciliate around its long body axis. In natural conditions the action of one of them prevails although the action of the other one may exist. The external factors (including Ni ions and rised medium viscosity) may evoke engaging of both mechanisms resulting in a periodical alternation of left and right spiralling.

A work hypothesis is proposed for explaining some peculiarities of the movement and hydrodynamics of ciliated protozoa. The main point of this hypothesis consists in the postulation that the movement of cilia occurs owing to a system of whirly streams produced by the ciliary system of protozoon. The ciliate body, in consequence, rotates along its body axis in a direction inverse to the direction of the normal whirl stream. The movement and spiralling of the ciliate depend not so much on its body shape as on the system of whirl streams which involve a compulsory flowing over the body surface of the surrounding liquid.

РЕЗЮМЕ

Опыты по влиянию ионов никеля и повышенной вязкости среды на фрагменты *Paramecium caudatum*, на интактных *P. caudatum* и *P. calkinsi*, а также на разные виды инфузорий, в том числе не имеющих перистомальной борозды, показывают, что направление вращения тела инфузорий вокруг продольной оси тела и инверсия направления этого вращения не зависят от формы тела простейших и определяются не гидродинамическими факторами, а иными причинами. По-видимому, у всех (или у многих ресничных инфузорий) есть два различных регуляторных механизма, обеспечивающих вращение простейшего вокруг продольной оси тела. В естественных условиях преобладает работа одного из них,

хотя может наблюдаться деятельность и другого. Внешние факторы (в том числе ионы никеля и повышение вязкости среды) могут вызывать включение обоих механизмов, в результате чего возникает периодическая смена лево- и правовращения.

Для объяснения некоторых особенностей движения и гидродинамики ресничных инфузорий предлагается рабочая гипотеза. Суть этой гипотезы заключается в том, что движение ресничных инфузорий осуществляется благодаря системе вихревых потоков, создаваемых ресничной системой простейшего. Тело инфузории, в результате, вращается вокруг продольной оси тела в направлении, противоположном кручению общего вихревого потока. Движение и вращение инфузорий очень мало зависит от формы тела, поскольку, благодаря системе вихревых потоков, осуществляется принудительное обтекание тела простейшего окружающей жидкостью.

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Stanisław DRYL

Response of ciliate protozoa to external stimuli

Reakcja orzęsków na bodźce zewnętrzne

In last decade the free-living ciliates became a classical model of experimental studies on motile phenomena of living matter on cellular and subcellular level. Electron-microscopic data on the fine structure of pellicle, flagella and cilia, combined with the new achievements in the field of physiology and biochemistry indicate important role of ionic composition of medium and ATP-ase — ATP system in excitation and contractile activity of those cellular components. However in spite of all these efforts, we are still far from being able to present an propriate theory on some basic phenomena involved in mechanics of three important forms of movement: ameboid, flagellar and ciliary one. Under these circumstances it is not surprising that our present theoretical approach to the problem of susceptibility of protozoan cell to external stimulation is also rather poor and inconsistent.

Protozoa possess the capacity of responding to a variety of physical and chemical stimuli by changes of direction of movement and rate of swimming. In response to local changes of environmental conditions the animals may respond with so-called "avoiding" reaction which may appear in more or less developed form, depending on the intensity of stimulation. It should be emphasized that all the motor response of protozoa is achieved without nervous system and in many cases even without existence of differentiated reception areas or locomotor organelles.

Parker 1919 put forward the hypothesis that the nervous system of metazoa could evolve from single cell showing already receptor and effector properties, which during long-lasting process of evolution got specialized in form or receptor cells and effector cells. Grundfest 1957, 1959, 1961 pointed out that in spite of above mentioned specialization, the excitable cells have retained the double function of receptor-effector system, possesing input, conductile and output components of action.

According to Grundfest the input component is chemosensitive but electrically not excitable, acting as transducer converting the energy of the specific stimulus into localized, non-propagated electrical response, which may have depolarizing or hyperpolarizing properties. The change of resting potential of cell membrane appears as local graded responses ("receptor potential") which may eventually (as so-called "generator potential") induce spike potentials in the conducting portion of excitable cell.

The conductile component is electrically sensitive but does not respond to chemical stimulation. Its main function is to conduct the impulse by means of spike potentials which are typical propagated, "all-or-none" responses, invariable in size and shape.

The output component is characterized by secretion of the chemical transmitter which may act upon the input component of another excitable cell.

The first attempt to apply this system to protozoa was done by D u n c a n 1967. According to him amoeba should be considered as a simple receptor-effector system with input and output components, but lacking the conductile portion.

Such system may exist in many other unicellular organisms, but it is the view of the author that many free-living ciliate protozoa possess all three components and this seems to be true at least in the case of *Paramecium*. This assumption is based on recent results achieved by K i n o s i t a, D r y l and N a i t o h 1964 a, b, c who were able to show that spontaneous, weak (not complete) reversal of ciliary beat in *Paramecium caudatum* is accompanied by graded depolarizing response while contraction of ectoplasm — by graded hyperpolarizing response (Fig. 1). However paramecia exposed to 1 mM soln. of Tris-HCl buffer medium containing appropriate concentration of barium and calcium chlorides responded with so-called "Periodic Ciliary Reversal"
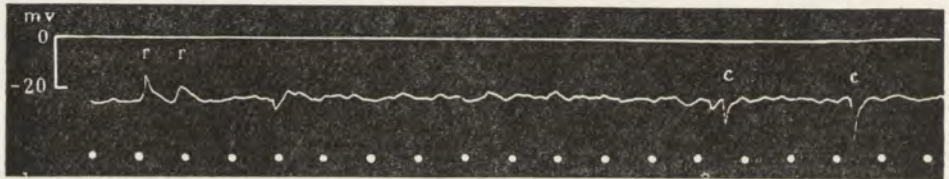
Fig. 1. Spontaneous change in membrane potential of *Paramecium* in an unstimulated condition. Note that the decrease or increase in inside-negativity of the membrane is correlated with the reversal (r) or contraction response (c) of the organism respectively. Measurement was done on *Paramecium* which had been kept for more than one hour in 2 mM $CaCl_2$ solution. Calibrated value of the electric potential of the tip of the intracellular microelectrode with reference to the indifferent electrode is given on the left side. Time signal: one second. (K i n o s i t a, D r y l and N a i t o h 1964 a)
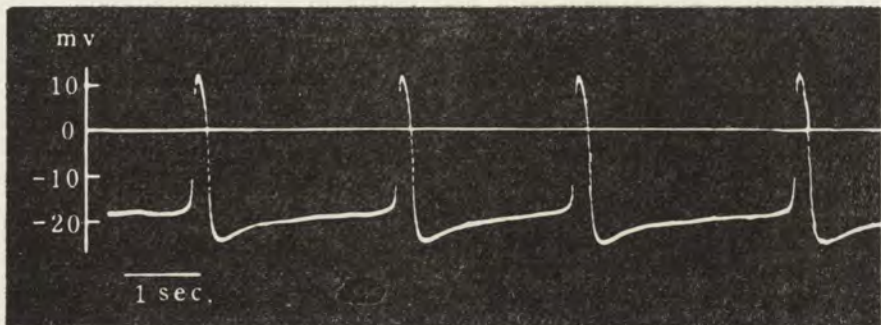

Fig. 2. Sponteneous action potentials in *Paramecium caudatum* during a periodic ciliary reversal (PCR) evoked by the mixture of 2 mM $BaCl_2$ + 1 mM $CaCl_2$. (K i n o s i t a, D r y l and N a i t o h 1964 a)

(D r y l 1961 b) i.e. short-lasting (0.5—1.0 sec.) reversal of ciliary beat followed by the normal forward movement, each response appearing alternately at approximately 1 second long intervals. As it is shown on the Fig. 2 each cycle of reversed beat of cilia was accompanied by "all-or-none" depolarizing spike which could be recognized as typical action potential similar in shape to analogus responses in other excitable cells. It looks like the cell membrane of *Paramecium* is the site for reception of external stimuli and generation of graded or propagated "all-or-none" responses. In other words input and conductile components are both localized within cell membrane of the ciliate whereas transmission of impulse to locomotor organells (cilia, cirri, myonemes) could correspond to output component of Grundfest's scheme (Fig. 3).
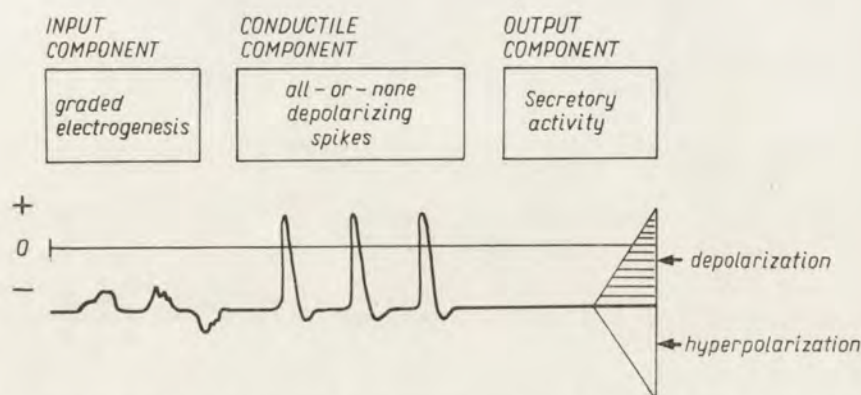
Fig. 3. Diagram showing relation between the output, conductile and output components of excitable cell and corresponding changes of intracellular potential. (Based on G r u n d f e s t's 1957 scheme)

There is strong evidence that in the ciliate protozoa Ca^{2+} and K^{1+} play essential role in excitation of ciliary beat and contraction of myonemes, whereas Mg^{2+} and Na^{1+} do not possess significant influence in this respect. It seems to be an important finding that all basic motor response in ciliates (chemotaxis, glavanotaxis, ciliary reversal, periodic ciliary reversal) can be elicited in medium devoid of sodium ions. Also Ba^{2+}/Ca^{+2} — induced depolarizing spikes (action potentials) could be developed in external medium free from Na^{1+} or any other monovalent cations and this casts serious doubt about possible role of so-called "sodium pump mechanism in generation of those responses. It is worth to mention that F a t t and K a t z 1953 reported action potentials of the crab muscle fibres to occur in solution without sodium ions. More recently H a g i w a r a, N a k a and C h i c h i b u 1964 supplied evidence that spike potentials in the giant muscle fibres of the barnacle *Balanus nubilis* appear in the absence of sodium in the external medium and this may suggest that those responses are determined by transmembrane movement of calcium and potassium ions only.

It was known for many decades that in the ciliate protozoa the motor excitability of the organism is detected as a short-lasting change of direction of ciliary beat (Jenning's "Avoiding reaction") or prolonged ciliary reversal (M a s t and N a d l e r 1926, O l i p h a n t 1938, M e r t o n 1923).

The significance of calcium/potassium factor for duration of ciliary reversal in *Paramecium caudatum* was demonstrated in the pioneer studies of K a m a - d a 1940 and K a m a d a and K i n o s i t a 1940. J a h n 1962, using the data of K a m a d a and K i n o s i t a, applied the Gibbs-Donnan principle to the established equilibrium between the surface of *Paramecium* and ionic composition of external medium; he has shown that duration of ciliary reversal remains constant when concentration of K^{1+} in the medium is proportional to the square root of Ca^{2+} concentration in accordance with the formula $= [K^{1+}]/\sqrt{[Ca^{2+}]} = $ const. J a h n suggested that reversal of ciliary beat of *Paramecium* may depend on the removal of calcium from binding sites of the cell membrane. G r ę b e c k i 1964, 1965, on the basis of experimental data achieved with agents causing external chelation and precipitation of calcium — suggested that the degree of excitation of *Paramecium* is related to the amount of calcium which remains at adsorption sites of cell membrane. His view was supported by K u ź n i c k i 1966 who analyzed the motor reactions of *Paramecium* caused by various cations (Cs^{1+}, Te^{1+}, Na^{1+}, K^{1+}, Ba^{2+}, Mn^{2+}, Sr^{2+}) at different level of external Ca^{2+} concentration and came to conclusion that the cations under study are acting by elimination of calcium ions from their binding sites in cortical layer of the ciliate.

N a i t o h and Y a s u m a s u 1967 showed that ^{45}Ca binding by *Paramecium* was inhibited by other cations present in external medium showing good agreement with Gibbs-Donnan principle. These calcium depleting effects of cations on the cell membrane were correlated with duration of induced ciliary reversal. The authors suggested that calcium ions liberated from the anionic sites of the cell membrane by an exchange reaction with other ions, induce reversal of ciliary beat. Recently it was demonstrated by N a i t o h (unpublished) that the glycerol-extracted models of paramecia respond with reversed ciliary beat when exposed to external medium containing ATP and calcium ions. This finding strongly supports the hypothesis of N a i t o h 1968 that calcium ions liberated from the cellular cation exchange system activate the contractile system energized by ATP.

It is still not clear what is the precise site of postulated cation exchange system releasing calcium ions. However, radioautographic studies on calcium binding in *Paramecium* suggest that ^{45}Ca accumulates at the surface of pellicle and it is interesting to state that G i b b o n s and R o w e 1965 found calcium-activated ATP-ase in the cellular fraction of *Tetrahymena* containing fragments of cilia.

S l e i g h 1969 brought evidence that excitability of contraction in *Spirostomum* is increased in media which contain appropriate concentration of: Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, oubain, digitoxin, caffeine, nicotine, iodoacetic acid, dinitrophenol and cyanide while it is reduced in medium free of calcium ions. It is suggested that the level of excitability depends on the concentration of free Ca^{2+} in the cytoplasm and that the above mentioned agents act on binding or that they cause an increase of free cytoplasmic Ca^{2+}.

Thus the control of ciliary reversal and contraction of myonemes in protozoa by calcium ions reminds of analogous role of these ions in triggering the activity of the muscle ATP-ase — ATP system. The liberated energy derived from splitting of ATP down to ADP is probably used for the activity of ciliary beat in similar way as it is utilized for contraction of myofibrilles

in muscle tissues of metazoa. The possible role of ATP-ase — ATP system in the excitability of protozoa seems to be confirmed by the fact that actomyosin-like protein complexes were extracted from *Paramecium* (van Wagten·donk and Vloedman 1951), *Tetrahymena* (Child 1959, 1961, Kamiya 1960, Watson and Hopkins 1962) phytoflagellates (Tibbs 1957, 1958, Jones and Lewin 1959) and *Amoeba proteus* (Simard-Duquesne and Couillard 1962 a, b, Guindon and Couillard 1964). It was shown that glycerin-extracted cilia and flagella proved to move in medium containing ATP (Hoffmann-Berling 1953, Brokaw 1958). Glycerin-extracted whole-cell models of protozoa (Hoffman-Berling 1953, Lepsi 1926, Levine 1956) respond with contraction to solution containing ATP. In this point it is worth to note that ATP — reactivated models of cilia and flagella show repeated contractions while the muscle models — single contractions only. It is suggested by Sleigh 1962 that this ability to show alternately contraction and relaxation depends on the specific contractile mechanism of cilia and flagella.

The ciliate protozoa can react by cessation of movement, contraction of body or "avoiding" reaction in response to mechanical stimulation. As a rule the motor reaction is alleviated or it may be even completely abolished when stimulation is repeated many times at short intervals (Wawrzyńczyk 1938, Kinastowski 1963 a). This phenomenon was very often explained in terms of physiological adaptation, "fatique" or "learning", although its real physiological basis is still obscure (Kinastowski 1963 b).

It was indicated by a number of authors that protozoa are more sensitive to external stimulation at the anterior end of body than at the posterior one and that in ciliates the region of the mouth is the most sensitive to chemical and mechanical stimuli (Naitoh 1958, Doroszewski 1961, 1962, 1963, Seravin 1962 b, Grębecki 1965). In favour of this idea speak also results achieved in the microsurgery experiments on some ciliates.

The important role of ions in external medium is evident from studies on susceptibility of protozoa to chemotactic stimuli. It was demonstrated by Dryl 1952, 1959 that cations could be arranged in the following sequence — according to intensity of their negative chemotactic action: $Ba^{2+} > K^{1+} > Mg^{2+} Ca^2 > Na^{1+}$. Similar range of cations was established for their depolarizing effects on resting potential of *Paramecium* (Kinosita, Dryl and Naitoh 1964 b) and for chemotactic action in *Stentor coeruleus* (Dryl and Pietrowicz-Kosmynka 1967). Moreover, it was shown recently by Pietrowicz-Kosmynka (personal communication) that chemotactic response of *Stentor coeruleus* obeys Gibbs-Donnan principle and consequently it may depend on competition of various cations with calcium for the same adsorption sites in the cell membrane in similar way as it was suggested by other authors in the case of continuous ciliary reversal induced by cations.

On general, *Stentor* proved to be more sensitive than *Paramecium* to chemotactic action of cations but was less sensitive to changes of external pH and chemotactic actions of lower alcohols. Experiments carried out with ten lower alcohols brought evidence that the stronger negative chemotactic action of alcohol is correlated with its higher molecular weight and that n-compounds are more active in this respect that iso (Dryl 1959 b). An intriguing similarity of these findings to smell sensitiveness towards alcohols in various groups of

animals should be emphasized (D e t h i e r and Y o s t 1952, M o u l t o n and E a y r s 1960).

As it was mentioned before, the duration of continuous ciliary reversal induced by potassium and calcium ions depends on their concentrations in external medium. The intensity of this motor response decreases with time, so that finally there is no ciliary reversal at all and at this stage the animals may recover completely — if stimulation is weak i.e. if potassium/calcium ratio — $[K^{1+}]/\sqrt{[Ca^{2+}]}$ is low or they may show very distinct slackening of the forward movement (sometimes remaining motionless) — when exposed to strong stimulation i.e. when ratio $[K^{1+}]/\sqrt{[Ca^{2+}]}$ is high. This "physiological" adaptation of ciliates to potassium-reach medium may cause complete inhibition of normal response of ciliate protozoa to chemical, mechanical and thermal stimuli. Some after-effect of potassium ions was indicated by series of experiments with paramecia exposed to potassium-reach medium (30 — — 40 mM soln of KCl) and then washed again in the medium devoid of potassium ions; for 2—3 minutes "washed" paramecia ceased to respond to strong negative chemotactic stimuli, whereas recovery of normal rate of swimming was noticed immediately after the change of environment (D r y l 1959 a).

Stentors adapted to potassium-reach medium show no detectable response towards chemotactic of mechanical stimuli (P i e t r o w i c z - K o s m y n k a, unpublished). Nevertheless their capacity of contraction is well preserved as it can be easy checked by puncture of body with the microneedle. It was proved that potassium-adapted stentors didn't respond to appropriate concentrations of nickel ions with contraction of body, which appears always in animals not adapted to potassium (D r y l, unpublished). It is suggested by the author that potassium ions may block some important physiological mechanism which is necessary for normal transmission of external impulse from the surface of cell to contractile myonemes apparatus in stentor.

The mechanism by which potassium ions inhibit the susceptibility of ciliates to external stimuli is unknown. The phenomenon might be realted to postulated effects of potassium ions on the movement of calcium within the cell membrane and induced depolarization of cell surface. The author belives that only extensive, joint studies on intracellular potential, cell permeability and movement of ions through cell membrane in different conditions of excitability of protozoan cell could elucidate this important problem.

Summary

The hypothesis is put forward that some ciliate protozoa (e.g. *Paramecium caudatum*) — like other excitable cells — retained the double function of receptor-effector system and possess the input, conductile and output component of action as postulated by G r u n d f e s t 1957.

The cell membrane of the ciliate protozoan is the site both for reception of external stimuli and their conduction, giving rise to graded or propagated "all-or-none" responses. Thus he input and conductile components are localized within the cell membrane of the ciliate whereas the transmission of impulse to the locomotor organelles (cilia, cirri, myonemes) could correspond to the output component.

The important role of some ions (Ca^{2+}, K^{1+}) and ATP-ATP-ase system for the excitation phenomena in the ciliate protozoa is discussed.

STRESZCZENIE

Przedstawiono hipotezę, w myśl której niektóre orzęski (np. *Paramecium caudatum*) — podobnie jak inne komórki pobudliwe — zachowały funkcje receptora-efektora i posiadają element „wejścia" (input), „przewodzenia" (conductile) oraz „wyjścia" (output) zgodnie z poglądem G r u n d f e s t a 1957. Błona komórkowa orzęska jest siedliskiem recepcji bodźca jak i jego przewodnictwa, umożliwiając pojawienie się reakcji „niepełnych" (graded responses) lub typu „wszystko lub nic". Tak więc elementy „wejścia" i „przewodzenia" są zlokalizowane w obrębie błony komórkowej orzęska podczas gdy przekazywanie bodźca do organelli ruchowych (rzęski, szczecie, myonemy) mogłoby być odpowiednikiem elementu „wyjścia".

W artykule podkreślono ważną rolę niektórych jonów (Ca^{2+}, K^{1+}) oraz układu ATP-ATP-aza w zjawiskach pobudzenia orzęsków.

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Some factors affecting the excitation
of contraction in *Spirostomum*

Certains facteurs affectant l'excitation de la contraction chez *Spirostomum*

Some years ago J. A. K i t c h i n g suggested to me that it might be interest-
ing to experiment on the effects of changes in ion concentrations on the thresh-
old of electrical excitation of contraction in *Spirostomum*. In some very preli-
minary experiments reciprocal changes in K and Na concentrations brought
about considerable changes in excitability, particularly in a solution with low
Na and high K concentrations, where the *Spirostomum* showed a sharp
decrease in excitability. Others working with K i t c h i n g have provided
valuable information about the movement and balance of ions between *Spi-
rostomum* and its surroundings. C a r t e r 1957 found that *Spirostomum* accu-
mulates K, the inside concentration being at least ten times that outside, and
that Br (substituted for Cl) was accumulated to a lesser extent; the internal
concentration of Na was maintained below that outside, especially at higher
concentrations of Na. The time of half exchange of the cell Na was about
3 minutes, and for K it was between 2 and 3 hours. The rate of uptake and the
internal concentration of K were considerably higher in fed than in starved
animals. *Spirostomum* also accumulates Ca to levels about 30 times that
outside (J o n e s 1966); an equilibrium concentration of internal Ca was reached
after 14 days, and the level reached was higher (up to a level of about 60 mM)
at higher external Ca concentrations; feeding caused an increase in the rate
of uptake and the final level of the internal Ca concentrations. A large fraction
of the Ca that enters is bound as calcium phosphate, so that even after 42 days
only some 50% of the ^{45}Ca is lost from animals loaded with the isotope.

The contraction of vertebrate striated muscle occurs when the concentra-
tion of Ca^{2+} around the myofilaments rises sufficiently for the myosin ATPase
to be activated. The resting level of calcium ions is in the region of $10^{-7}M$,
and only needs to be raised to about $10^{-6}M$ in order for contraction to occur.
The relaxation of muscle is brought about by the active uptake of Ca into
storage sites in the endoplasmic reticulum (sarcoplasmic reticulum) to return
the Ca^{2+} concentration to the resting level. A release of Ca^{2+} from storage
sites occurs when the muscle is stimulated by the nerves or by a direct
electrical stimulus, and may also be induced by a number of other treatments,
e.g. a raised K concentration, or caffeine. The oscillatory changes in length of
insect fibrillar muscle are not synchronized with any form of nervous excita-
tion, and J e w e l l and R u e g g 1966 have found that oscillations of glycerol

extracted fibrillar muscle could be activated and controlled by a variation of the Ca^{2+} concentration, the threshold for activation being in the region of 10^{-7} to 10^{-8} M Ca^{2+}.

There is an increasing body of evidence that Ca^{2+} is required for the activation of the ATPase enzymes in a variety of contractile activities of Protozoa, including the contraction of cilia of *Tetrahymena* (G i b b o n s 1965) and the contraction of glycerol extracted models of *Vorticella* stalk myonemes (H o f f m a n n - B e r l i n g 1958). N a i t o h 1968 has recently put forward the hypothesis that the reversal of ciliary beating of *Paramecium* is the result of a Ca-induced contraction, and J o n e s, J a h n and F o n s e c a 1966 have found that the rate of shortening during the contraction of *Spirostomum* is reduced in Ca-free solutions.

J a h n 1966 has observed that no action potentials of the type found in muscle have been reported in Protozoa, and we (J a r m a n and S l e i g h, unpublished observations) are able to corroborate this statement for the ciliates *Stentor* and *Spirostomum*, for in careful electrophysiological studies of these ciliates no changes in potential were observed to accompany contractions of the animals. Electrically induced contractions of *Spirostomum* also differ from those of vertebrate striated muscle in that the myoneme contractions begin at the anodal end of the ciliate, while the contraction of an electrically stimulated muscle starts at the cathodal end. Excitation of both types of structure has been explained by J a h n 1966 as the result of the association of Ca^{2+} with the contractile proteins; in both types Ca^{2+} will associate with cytoplasmic proteins more quickly at the anodal end of the structure, but in the muscle with an excitable membrane large quantities of Ca^{2+} will be released at the cathodal end and initiate the contraction there.

Spirostomum is believed to shorten as a result of an active contraction of "myoneme" proteins. B a n n i s t e r and T a t c h e l l 1968 have made the attractive suggestion that in the related heterotrich ciliate *Stentor* the filamentous 'M' fibres are responsible for the contraction of the body while the active extension may be brought about by the microtubular 'km' fibres. In this study we are primarily interested in the M fibres, which in *Stentor* are mainly composed of filaments 80—100 Å in diameter, the bundles of which are not completely surrounded by membranes, and are often close to mitochondria. No membranes may be seen associated with the M fibres of *Spirostomum* in the electron micrographs of F i n l e y, B r o w n and D a n i e l 1964, but mitochondria and membrane-bound vesicles are often found close to the fibres; the filaments of fibres in *Spirostomum* were found by Y a g i u and S h i g e n a k a 1963 to be about 40 Å thick.

The manner in which the filaments within M fibres interact to cause shortening is unknown, but is is likely — by analogy with the contraction of the myoneme of the *Vorticella* stalk — that a Ca^{2+} — sensitive ATPase is involved. The observations reported here support this hypothesis.

Methods

The *Spirostomum ambiguum* used in these experiments were cultured either in Chalkley's medium or Carter's medium (compositions shown in Table 1), with rotting wheat grains to provide an abundant growth of food organisms for the ciliates. In experiments involving changes in the ionic concentration

of normal constituents of the medium around the *Spirostomum*, ciliates from the cultures were equilibrated for at least 12 hours in fresh samples of the medium in which they had been cultured. In some experiments test substances were dissolved in special media in which the concentrations of the common ions of the culture solutions were modified; the compositions of these media are also shown in Table 1.

Table 1

The constitution of media used in the experiments (in mM)

	Chalkey's medium			Carter's medium			
	Normal	6×10^{-5}M Ca^{2+}	0.5×10^{-5}M Ca^{2+}	Normal	No Ca^{2+}	No K^+	Li^+ Medium
NaCl	1.4	1.4	1.4	2.0	2.0	2.5	—
KCl	0.55	0.55	0.55	0.5	0.5	—	0.5
LiCl	—	—	—	—	—	—	2.0
NaHCO$_3$	0.045	0.045	0.045	—	—	—	—
CaCl$_2$	0.025	0.055	—	0.5	—	0.5	0.5
MgCl$_2$	—	—	—	0.2	0.7	0.2	0.2
Ca H$_4$ (PO$_4$)$_2$	0.005	0.005	0.005	—	—	—	—
K$_2$H PO$_4$	—	—	—	0.1	0.1	—	0.1
Na$_2$HPO$_4$	—	—	—	—	—	0.1	—
KOH	—	—	—	0.01	0.01	—	0.01
NaOH	—	—	—	—	—	0.01	—

The experiments discussed here are of two types, the first investigating the excitation of contraction in *Spirostomum* by an electrical discharge from a capacitor, and the second investigating spontaneous excitation of contractions in various media. Excitability by electric stimulation was studied by placing groups of ciliates (usually 10) in a perspex chamber 1 cm square and 2.5 mm deep, across two opposite sides of which were silver/silver chloride electrodes; the proportion of ciliates which contract when a charged capacitor was allowed to discharge through the medium between the electrodes was taken as a measure of the excitability of the ciliates. Fig. 1 shows the percentage of ciliates contracting when the capacitor was charged to various levels of potential difference. In the subsequent experiments reported here the voltage was fixed at that which would cause 50% of the untreated (control) animals to contract, and the excitability of samples of control ciliates and of ciliates from experimental solutions was measured at intervals throughout the experiments, ciliates which had been subjected to electrical stimulation being discarded after each test.

In a number of solutions *Spirostomum* was found to contract spontaneously. To measure the intensity of this spontaneous excitation and follow its time--course, a very small drop of culture medium containing 10 animals was pipetted into a solid watchglass and about 2 ml of the test solution was added. The animals were then watched under a low power microscope, and the number of ciliates contracting in each minute was recorded until a stable level was reached.
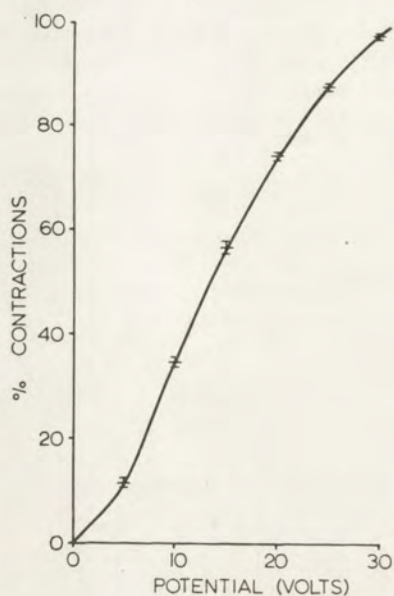
Fig. 1. The percentage of ciliates which contracted at various voltages in the chamber described in the text. Each point represents the mean of 100 tests, each involving 10 animals; the vertical lines indicate the standard error of the mean

All of the chemicals and drugs used were obtained from British Drug Houses, with the exception of Digitoxin, which was obtained from the California Corporation for Biochemical Research. The culture media were made with Analar chemicals with known impurity limits.

Results

1. Excitiation by electrical stimuli

a. At various concentrations of some divalent cations

The changes in the percentage contraction of *Spirostomum* samples following transfer to Chalkley's medium containing added $CaCl_2$ and no $CaCl_2$ are shown in Fig. 2. The temporary increase in excitability found in a Ca-rich solution is also found in media containing $BaCl_2$, $MgCl_2$, and $MnCl_2$ at concentrations comparable with those of the calcium in the medium (Fig. 3). The excitation decreases markedly within an hour or so in media with little or no Ca. The effects of $CuCl_2$ and $NiCl_2$ are quite different from those of the other divalent cations shown in Fig. 3; they are toxic within a couple of hours at concentrations well below the Ca concentration of the medium. Both ions, however, make *Spirostomum* more excitable to electrical stimulation at much lower concentrations, and in $CuCl_2$ especially the ciliates contract spontaneously (See 2 a).
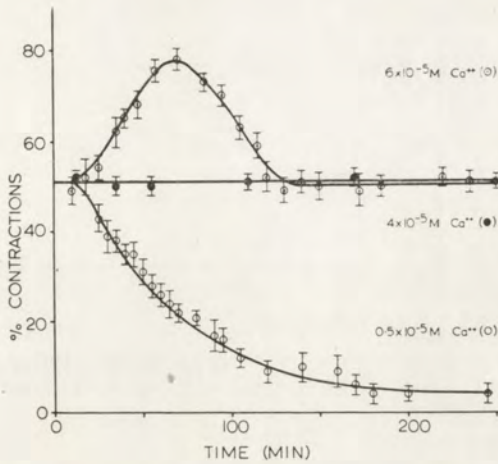
Fig. 2. The percentage of ciliates which contracted at a fixed voltage of condenser discharge at various times following transfer to a medium with added $CaCl_2$ (6×10^{-5}M Ca^{2+} in the test solution ⊙), a medium with no $CaCl_2$ (0.5×10^{-5}M Ca^{2+} in the test solution ○) and normal Chalkley's medium (4×10^{-5}M Ca^{2+} in the test solution ●). The vertical lines indicate the standard error of the mean
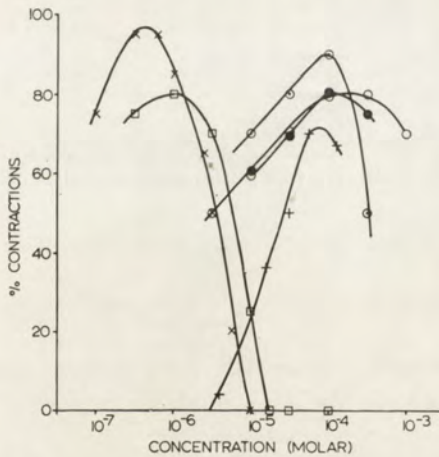
Fig. 3. The percentage of ciliates which contracted at a fixed voltage in solutions of the chlorides of divalent ions of various molarities. The symbols represent × Cu, □ Ni, ⊙ Ba, ○ Mn, ● Mg and + Ca

b. In the cardiac glycosides digitoxin and ouabain

The excitability of *Spirostomum* is increased at low concentrations of digitoxin (Fig. 4); an increase in the percentage of contractions has been found at concentrations as low as 10^{-10}M. Similarly, in solutions of ouabain (strophanthin — G) the excitability is increased, the rise in percentage of animals contracting being progressively delayed with greater dilution of the glycoside (Fig. 5). When animals were placed in ouabain solutions containing modified

4*
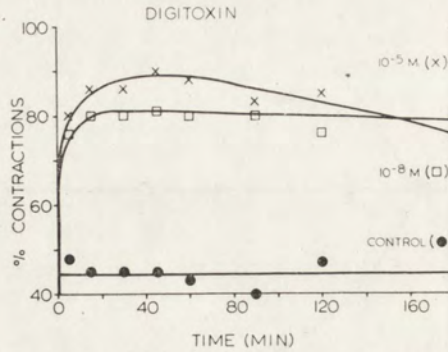
Fig. 4. The percentage contractions at a fixed voltage following transfer of the ciliates to solutions of digitoxin
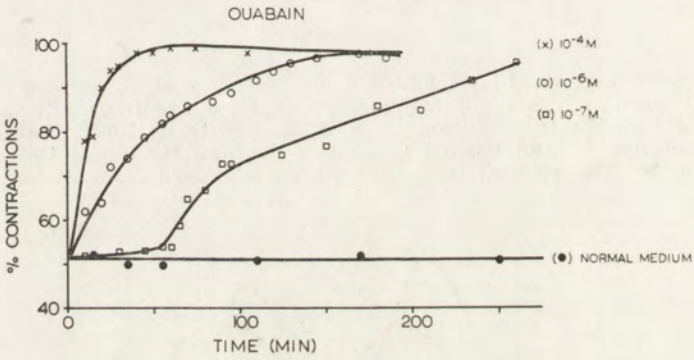
Fig. 5. The percentage contractions at a fixed voltage following transfer of the ciliates to solutions of ouabain
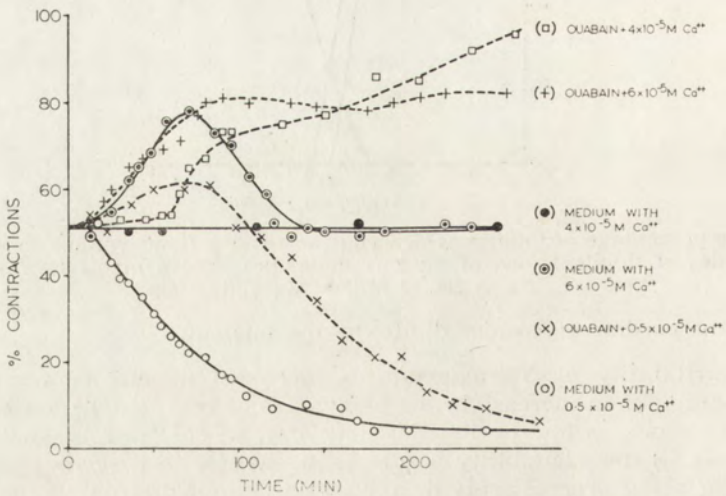
Fig. 6. The percentage contractions at a fixed voltage following transfer of the ciliates to solutions with 10^{-7} M ouabain (dotted lines) and without ouabain (full lines) at three calcium concentrations

calcium concentrations, it was found (Fig. 6) that the fall in excitability in the low-Ca solution was delayed by ouabain, and the rate of rise of excitability in ouabain is accelerated in the Ca-rich solution.

2. Spontaneous excitation by "chemical stimulation"

a. In polyvalent cations

Spontaneous contractions occurred at a very high rate in a 1 mM solution of $CuCl_2$, but the ciliates died within 15 minutes; at lower concentrations of $CuCl_2$ the spontaneous contractions were less frequent but persisted for a longer time (Fig. 7) — the *Spirostomum* in a $10^{-7}M$ solution were still swimm-
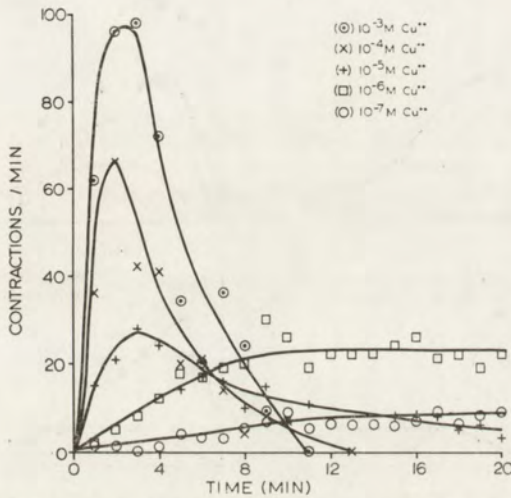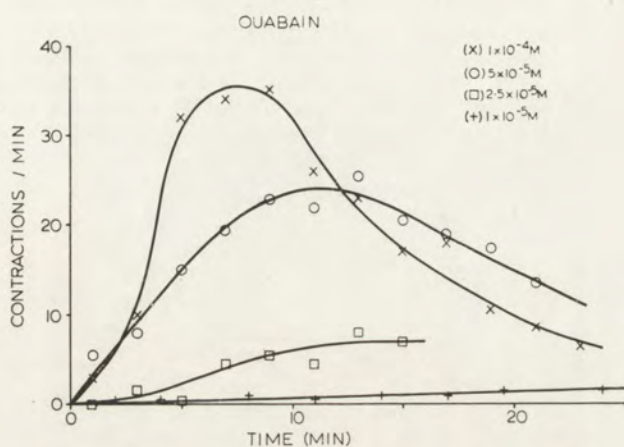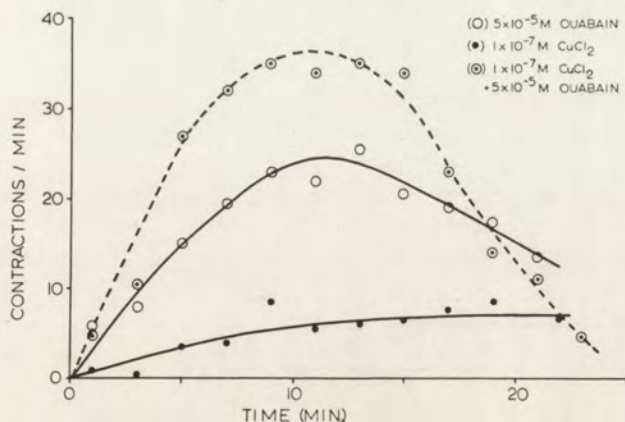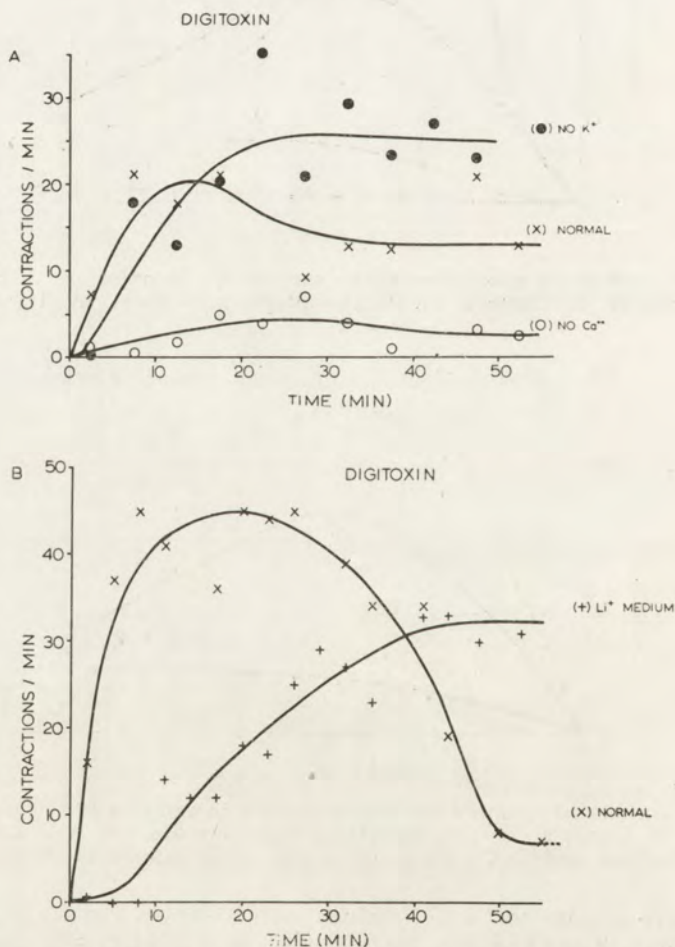

Fig. 7. The number of spontaneous contractions in samples of 10 animals following transfer to Carter's medium containing various concentrations of $CuCl_2$
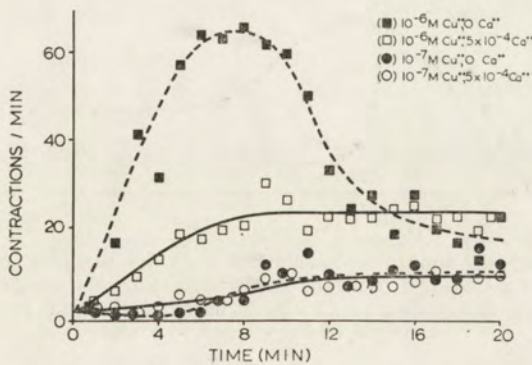
Fig. 8. The number of spontaneous contractions in samples of 10 animals following transfer to solutions of $CuCl_2$ in normal Carter's medium (solid lines) and in Carter's medium without Ca (dotted lines)

ing actively and showing a few spontaneous contractions after 80 minutes. When the $CuCl_2$ was dissolved in Carter's medium without Ca, the rate of excitation in $10^{-6}M$ $CuCl_2$ was higher than at the same copper concentration in normal medium (Fig. 8); the rate of spontaneous contractions at $10^{-7}M$ $CuCl_2$ was similar in normal and no-Ca media.

In $ZnCl_2$, $FeCl_3$ and $HgCl_2$ solutions spontaneous contractions occurred at higher rates in more concentrated solutions, and at a higher rate with a given test concentration when the metallic chloride was dissolved in a no-Ca medium (Fig. 9). The ionic concentration required to provoke spontaneous contractions
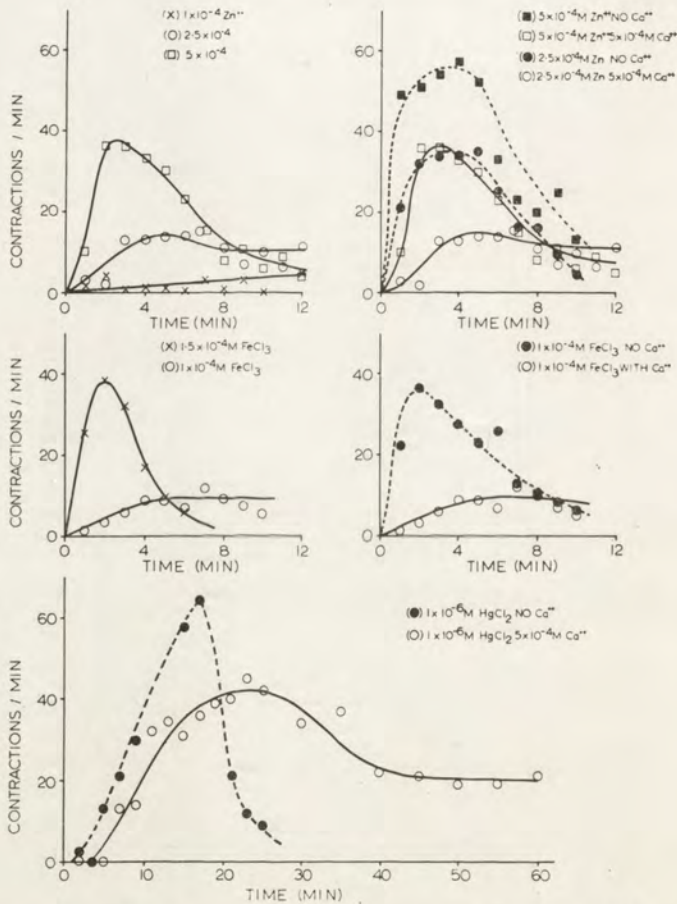
Fig. 9. The number of spontaneous contractions in samples of 10 *Spirostomum* following transfer to Carter's medium containing various concentrations of $ZnCl_2$, $FeCl_3$ and $HgCl_2$ in the presence of Ca (crosses and open symbols), and in the absence of Ca (closed symbols)

was higher in $FeCl_3$ and $ZnCl_2$ than in the very toxic $HgCl_2$, and in all three of these salts contractions occurred within a narrower concentration range than that found with $CuCl_2$.

b. By cardiac glycosides

Only high concentrations of ouabain caused frequent spontaneous contractions of *Spirostomum* (Fig. 10), and the occurrence of contractions is delayed for several minutes in the more dilute solutions. The shapes of the lines drawn

Fig. 10. The number of spontaneous contractions in samples of 10 *Spirostomum* following transfer to Carter's medium containing ouabain at 4 concentrations

Fig. 11. The number of spontaneous concentrations in samples of 10 animals following transfer to Carter's medium containing only ouabain (O), only $CuCl_2$ (●) and both ouabain and $CuCl_2$ dissolved in the same sample of medium (◎)

in Fig. 10 are similar to those obtained with Cu^{2+} in Fig. 7. The effects of Cu^{2+} and ouabain are additive, for, as shown in Fig. 11, a solution containing both ouabain and $CuCl_2$ caused more contractions than either substance separately.

The digitoxin used was considerably less soluble than ouabain, the solutions used in the experiments summarized in Fig. 12 are believed to have been satu-

rated solutions of digitoxin made up in the various media; those in group A were all made up at the same time under identical conditions so that they are comparable. The spontaneous contractions which occur in digitoxin reach a higher level in a potassium-free medium than in normal Carter's medium, and remain at a low level in a no-Ca medium. The results shown in Fig. 12 B were from experiments carried out on better-fed animals from a different culture, but again the drug solutions in the two media are exactly comparable. The effect of digitoxin on these well-fed animals is distinctly larger than that shown in Fig. 12 A, but in the medium in which sodium is replaced by lithium, the rise of excitability is delayed and does not reach such a high level as that found in the normal medium.

Fig. 12. The number of spontaneous contractions in samples of 10 animals following transfer to Carter's medium containing 1 mM digitoxin ($\times$), the same concentration of digitoxin in a no-K medium (●), 1 mM digitoxin in a no-Ca medium (O) and 1 mM digitoxin in a Li medium (+). Graphs A and B relate to two sets of experiments done on animals from different cultures

c. By caffeine and nicotine

Solutions of caffeine cause spontaneous contractions over a wide range of concentrations (Fig. 13), the rise of excitability being progressively delayed with increased dilution of the drug. It is very noticeable that in caffeine the animals extend very slowly after contraction, particularly at the higher concentrations. Nicotine causes similar prolonged contractions at a high frequency (Fig. 14). Solutions of nicotine made up in a medium without calcium tended to show a delayed rise in excitability, but reached a frequency of contractions similar to that found in the normal medium.
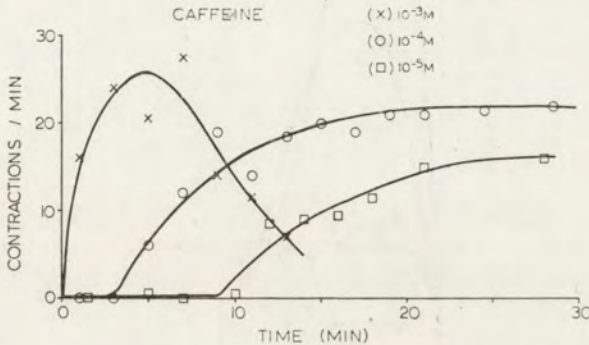
Fig. 13. The number of spontaneous contractions following transfer of samples of 10 animals to caffeine solutions of various concentrations
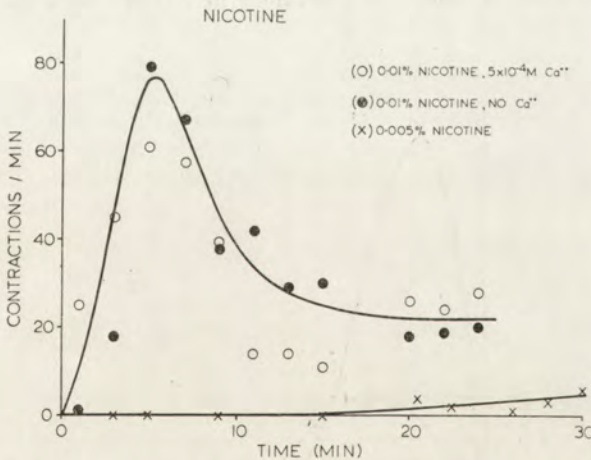
Fig. 14. The number of spontaneous contractions following transfer of samples of 10 animals to 0.01% (O) and 0.005% (✕) nicotine solutions in Carter's medium, and to a 0.01% nicotine solution in a medium without Ca (●)

d. By respiratory inhibitors

Several respiratory inhibitors were found to cause spontaneous contractions of *Spirostomum*. Iodoacetic acid (I.A.A.) and 2,4-dinitrophenol (D.N.P.) were both active at reasonably low concentrations (Figs 15 and 16), and in both
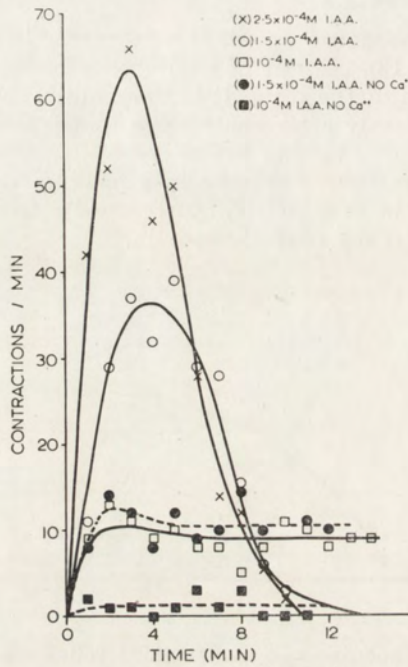
Fig. 15. The number of spontaneous contractions following transfer of samples of 10 animals to iodoacetate solutions in Carter's medium
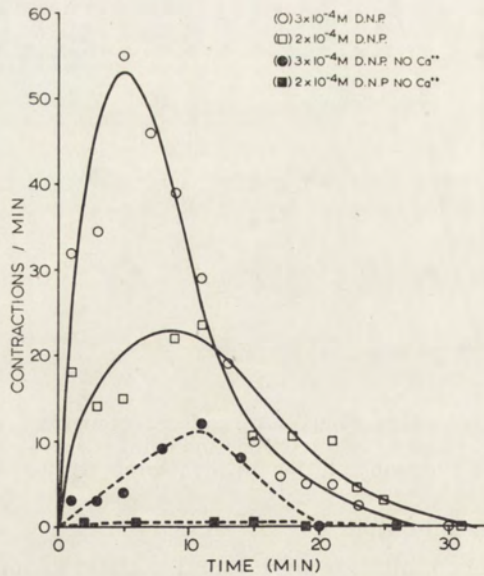
Fig. 16. The number of spontaneous contractions following transfer of samples of 10 animals to 2,4-dinitrophenol solutions in Carter's medium

cases solutions made up in a calcium-free medium showed a lower excitability than those in the normal medium. Sodium fluoride did not cause contractions, even at a concentration of 10^{-2}M; in this solution the ciliates were still swimming and were able to contract on mechanical stimulation after 30 minutes. Sodium cyanide caused an immediate contraction at concentrations of 3 to 10×10^{-3}M, and the animals died within minutes without showing more than one or two further contractions. In 1 to 2×10^{-3}M sodium cyanide more frequent spontaneous contractions were seen (up to 25/minute in 10 animals) in the first five minutes in the solution, but only occasional contractions were seen after this time altough the animals were still able to contract and were still swimming after 2 hours.

Discussion

Spirostomum actively accumulates Ca and converts a considerable part of the Ca^{2+} taken up to insoluble calcium phosphate; this probably constitutes that fraction of the Ca content which is not exchanged within 42 days. A further large fraction of the internal Ca is only slowly exchanged and is held to be more or less tightly bound in the cytoplasm or its organelles. Only a small part of the internal Ca is freely exchangeable. These three fractions constitute the fixed, bound and free components of Ca in *Spirostomum* suggested by J o n e s 1967. There is evidence that Ca and phosphate may be accumulated by mitochondria, in an active transport process which is capable of reducing the Ca^{2+} concentration of the surrounding fluid to 10^{-6}M in in vitro experiments, and that in the organelles calcium hydroxyapatite is formed by a process of oxidative phosphorylation in which Ca^{2+} is substituted for ADP in reactions associated with the electron transfer chain (L e h n i n g e r 1967). In muscle cells Ca^{2+} is actively accumulated within the membrane-bound vesicles of the sarcoplasmic reticulum, so that the Ca^{2+} concentration of the cytoplasm is reduced to very low levels (E b a s h i and L i p m a n n 1962, W e b e r, H e r z and R e i s s 1964). It is suggested that similar mechanisms involving the accumulation and binding of calcium in mitochondria and perhaps in vesicles of the endoplasmic reticulum occur in *Spirostomum*, so that the cytoplasmic concentration of Ca^{2+} is maintained at a low level.

An attempt has been made in Fig. 17 to show some of the possible interactions in this system, as they involve Ca^{2+} and its availability to excite contractions. It is necessary to consider the pattern of exchange of Ca^{2+} between the cytoplasm and the outside medium, and between the cytoplasm and temporary binding sites (unspecified, but probably in the vesicles of endoplasmic reticulum or in the mitochondria); also there is an uptake of Ca^{2+} from the cytoplasm to form calcium phosphate, presumably in the mitochondria. The way in which these three aspects of Ca^{2+} distribution may be affected by the treatments described in this paper will be considered separately. The present state of knowledge concerning the role of Ca^{2+} in the muscle of metazoan animals has been reviewed by S a n d o w 1965, Daniel 1965, C a l d - w e l l 1968 and L a n g e r 1968.

It is clear from Figs. 2 and 3 that the external Ca^{2+} concentration influences the excitability of *Spirostomum*, as it does in a comparable manner in heart muscle (R i n g e r 1883) and striated muscle (L ü t t g a u 1963), and it
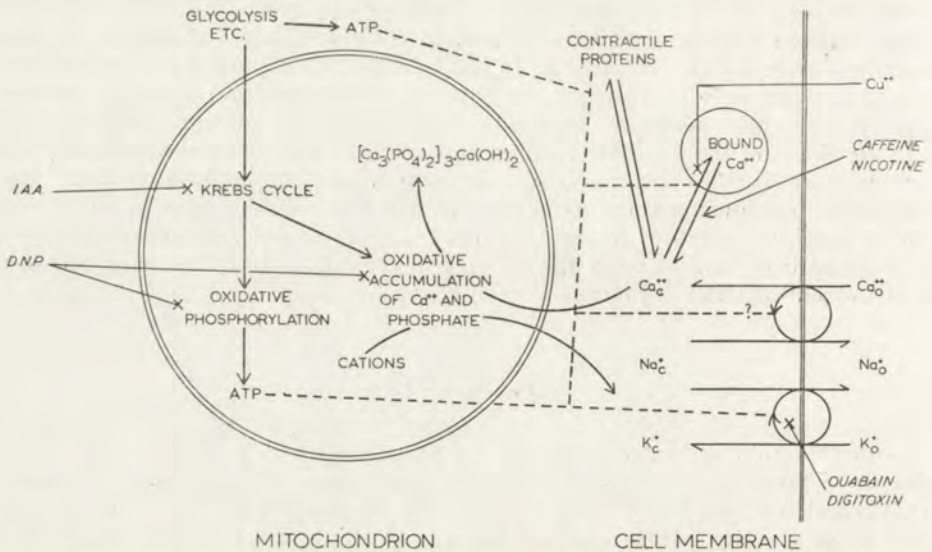
Fig. 17. Scheme to show the distribution of Ca^{2+} and its relation to the other cellular activities discussed in the text. Ca_0^{2+}, K_0^+ and Na_0^+ represent the ions in the outside medium, and Ca_c^{2+}, K_c^+ and Na_c^+ represent the ions in the cytoplasm. The activities which are believed to occur within mitochondria are shown at the left in a double circle, and the outside membrane is at the right. The bound Ca^{2+} is probably associated with the vesicles of the endoplasmic reticulum, and its uptake may involve another active transport ATPase system

may be assumed that this influence is exerted through an effect on the internal Ca level, by exchange of Ca^{2+} between the cytoplasm and the external medium. The divalent ions of Ba, Mn and Mg may replace Ca in some of its functions and so may raise the cytoplasmic level of Ca^{2+} available for excitation of contractile proteins. Cu and Ni appear to have a different effect at a much lower concentration, and will be considered later.

Cardiac glycosides, including ouabain and digitoxin, are known to act on the ATPase activity associated with the active transport of K^+ and Na^+ (such transport is believed to account for the distribution of these ions in *Spirostomum* mentioned earlier), but have not been found to have a direct effect on Ca^{2+} transport ATPases (S k o u 1965, G i b b s, R o d d y and T i t u s 1965); in the presence of these glycosides an accumulation of cytoplasmic Na^+ may occur, which may be the exchange currency required for the intake of Ca^{2+} (C a l d w e l l 1968). This explanation is supported by the relations between ouabain action and calcium concentration (Fig. 6), and between digitoxin action in a medium containing no Ca, where the response is reduced, in a lithium medium, where it is delayed but still large, and in a no-K medium where the excitability is increased (Fig. 12). B a k e r, B l a u s t e i n, H o d g k i n and S t e e n h a r d t 1967 concluded that a stimulation of inward movement of Ca into nerve axons took place when the internal Na concentration was raised or the external Na concentration was reduced.

The action of cardiac glycosides applied to intact cells seems to be limited to the transport systems of the exterior surface membrane. Certain toxic

metal ions also inhibit activity of ATPase systems, including those involved in the transport of ions across membranes; G i b b s, R o d d y and T i t u s 1965 found that Cu^{2+}, Zn^{2+} and Hg^{2+} abolished ATPase activity of membranes, and P e t e r s, S h o r t h o u s e and W a l s h e 1965 concluded that an excitatory effect of Cu^{2+} may be due to the inhibition of an ATPase of intracellular membranes. In this study it was found that in media with no Ca the excitatory effect of Cu^{2+} and other metallic ions is enhanced, especially at higher concentrations (Figs 8 and 9); the absence of Ca may increase the inward movement of toxic ions to active sites, but it seems unlikely that the main effect of Cu is on any ATPase in the membrane at the cell surface because excitability would in that case be reduced in a no-Ca medium. It is therefore concluded that Cu may act on mechanisms concerned with the accumulation of Ca^{2+} into organelles (mitochondria or endoplasmic reticulum) within the cytoplasm, where the Ca is temporarily or permanently bound; the additive effect of the excitation by ouabain and by Cu reinforces the view that these two actions are at different sites.

The contraction of muscle is potentiated by a low concentration of caffeine, which is believed to cause a release of bound Ca^{2+} and an inhibition of uptake of Ca^{2+} by sarcoplasmic reticulum (B i a n c h i 1961, Sandow 1965); nicotine may release Ca^{2+} in the same way as caffeine (A h m a d and L e w i s 1962, N a y l e r 1963). The increase in excitability of *Spirostomum* in caffeine and nicotine (Figs 13 and 14) may also be explained as an increase in cytoplasmic Ca^{2+} resulting from a release of bound Ca^{2+}; it would not be expected that this release of Ca^{2+} was affected by the external Ca concentration, except perhaps in the very early stages of the action in a no-Ca medium, when some free Ca might be lost to the outside rather than affecting the contractile proteins.

A dissociation of the mechanical response of muscle from the action potential may occur in solutions of (1) iodoacetate, (2) 2,4-dinitrophenol and (3) cyanide (S a n d o w 1965). The effects of these poisons on respectively (1) the Krebs cycle, (2) the intermediate and (3) the terminal stages of the electron transfer system of oxidative phosphorylation are well known, but more specific effects than those which cut off the supply of ATP are also suspected in *Spirostomum*, since the ability to swim and contract is retained after a very considerable change in excitability has taken place. One possibility is the poisoning of the Ca^{2+} uptake mechanism of mitochondria, for V a s i n g t o n and M u r p h y 1962 found that some poisons including D.N.P. and cyanide inhibited Ca^{2+} uptake by rat kidney mitochondria, while sodium fluoride did not have an inhibitory action. Accumulation of Ca^{2+} into mitochondria may be a means whereby the cytoplasmic Ca concentration is normally maintained at its low resting level. The sensitivity of the effects of I.A.A. and D.N.P. to external Ca concentration suggests either an action at the surface membrane or an action on the Ca equilibrium in the superficial cytoplasm; however, D.N.P. does not affect Na^+ and K^+ transport at concentrations above those used here (S k o u 1965), and it does not affect the uptake of Ca^{2+} into vesicles of liver cell endoplasmic reticulum, but it does inhibit the accumulation of Ca^{2+} by mitochondria in the same cells (C a r a f o l i 1967).

It is concluded that the contractile machinery of *Spirostomum* is activated by a rise in the cytoplasmic concentration of Ca^{2+}. The binding of Ca^{2+} in cytoplasmic organelles, probably in endoplasmic reticulum vesicles, and uptake

and fixation of Ca in mitochondria, maintain the Ca^{2+} concentration of the cytoplasm at a low level, probably very considerably below the Ca^{2+} concentration of the outside medium. Excitation is probably normally brought about by an increase in the permeability of the cell surface membrane to Ca^{2+} and a consequent inflow of Ca^{2+} sufficient to activate the ATPase of the contractile proteins; further release of Ca^{2+} from the binding sites could be involved but does not seem to be essential.

Summary

Quantitative estimates have been made of the excitability of the contraction mechanism in *Spirostomum* by counting the number of animals which respond to a standard electric shock, or by assessing the rate of spontaneous excitation. Excitability is increased in media containing certain concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, ouabain, digitoxin, caffeine, nicotine, iodoacetic acid, dinitrophenol and cyanide, and is reduced in a medium lacking Ca^{2+}. Excitability is believed to depend on the concentration of free Ca^{2+} in the cytoplasm, and the action of all of the excitatory agents named is believed to cause a rise in the level of free cytoplasmic Ca^{2+} by effects on the binding or transport of calcium.

RÉSUMÉ

On a estimé quantitativement l'excitation du mécanisme de contraction chez *Spirostomum* en comptant le nombre d'animaux qui répondent a un choc éléctrique standarisé ou bien en mesurant la velocité de l'excitation spontanée. L'excitabilité est augmentée dans des milieux contenant certaines concentrations de Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, ouabaine, digitoxine, cafféine, acide iodoacétique, dinitrophénol et cyanure, et est reduite dans un milieu sans Ca^{2+}. L'excitabilité dans le cytoplasme et l'action de tous les agents excitants nommés causent, selon l'auteur, une augmentation du nivau de Ca^{2+} cytoplasmatique libre en ayant un effet sur la liaison ou transport du calcium.

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Note added in proof

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Responses of the ciliate *Dileptus* to mechanical stimuli

Reakcja orzęska *Dileptus* na bodźce mechaniczne

The aim of the present communication has been to supplement the material of facts concerning the localization of reactivity in the ciliate cell. The results of investigations carried out for several years on *Dileptus* are to be presented in an abbreviated form. I do not intend to treat the problems from a theoretical aspect either to analyse the mechanism of the ciliary movement. I tried to select the most simple reactions of ciliates, as withdrawal and the reaction of the forward start which was as yet rarely investigated. The occurrence of those two reactions served to my description of a certain model of the behaviour of *Dileptus*. It should be however noted that I had in view only a simplified image of reactivity and not creation of a theory of its mechanism. The experimental problem was to analyse the character of motor reactions and the localization of sensitive areas on the surface of the ciliate body.

Mechanical stimuli have been chosen for the study because they may be applied locally. Out of them touching, puncture and bisection of the ciliate cell were experimented. As supplementation of those studies, a mechanical stimulus acting upon the whole body surface i.e. water shake was applied. A special material fit for those investigations was selected.

The reactivity of *Dileptus* is comparatively little known and the considerable dimensions of this ciliate simplify the procedure of experiments. Essential is its high ratio of body length to its width which makes possible to study the phenomena along the long body axis. Another important property of this ciliate is the position of cytostome near the anterior end of the body and the extension of the buccal ciliature along proboscis.

Presently the studies on morphogenesis and regeneration in *Dileptus* are carried out. In this communication only the results of the study on its behaviour are reported although both fields of research encroach upon each other.

The major part of the results reported had been gained in the course of the work on regeneration and division.

The whole investigation on *Dileptus* is being summarized in the form of a monography of this ciliate.

As introduction to the present communication, a short characteristic of *Dileptus* is to be presented because this genus is comparatively scarcely known.

The majority of experiments were carried out on the species determined as *Dileptus cygnus* Clap. et L. Some results gained previously on *Dileptus anser* O.F.M. are also reported. The above determinations are based on the taxonomic and morphological studies of *Dileptus* by D r a g e s c o 1965.

The cultures of both species originated from individuals taken from natural stations in the surrounding of Warsaw, from pools near Królikarnia and Mila-nówek. As food for the *Dileptus* cultures, small ciliates ware used. After having overcome the initial difficulties with the culture associated with application of wild material, a gradual standarization of the culture conditions was achieved in the course of progressing work. *Dileptus anser* was fed with colpidia. As food for those ciliates, powdered milk in definite proportion was used. The next step for standarization of food was the application of axenic cultures of *Tetrahymena pyriformis* cultivated on pepton nutrient of Difco. This food however proved to be of a not full value and colpidia cultivated on milk had to be administered at definite intervals of time. As culture medium the buffer of Dryl or the Pringsheim's solution were applied.

Dileptus cygnus was fed with colpidia and *Tetrahymena* which — in turn — were cultivated on dry yolk. As medium served the Pringsheim's solution. No essential differences involved by the mode of feeding were observed in the behaviour of ciliates.

For eliminating the differences in the results of experiments involved by the degree of saturation with food, individuals — deprived of food — were kept for 24 hrs. in the Dryl's buffer or in Pringsheim's solution. This involved also the absence of dividing individuals and made the ontogenetic phase of ciliates more or less uniform. In the case of experiments on dividing indivi-duals, the culture of *Dileptus* had been fed for 24 hrs. prior to the experiment.

The body dimensions of both species under study vary in a broad range and show a specific polymorphism which has been reported by a number of authors (e.g. V i s s h e r 1923). The individuals with body length amounting approx. 1 mm were selected for experiments.

Proboscis is located on the anterior part of the cell. At its base is the round orifice of cytostome. The cytological structure of proboscis has been reported by several authors (V i s s h e r 1927, P e s c h k o w s k y 1931, G e l e i 1927, J o n e s 1951, D u m o n t 1961, D r a g e s c o 1963). On the base of their results, the following structures may be distinguished: feeding cilia, normal cilia, and structures called by G e l e i "Sinnesstiftchen".

On the oral aspect of proboscis, a band of trichocysts may be observed. D u m m o n t 1961 revealed by electron microscopy fibrils running from the basal bodies of feeding cilia and united into one stem on the oral sector of proboscis. The cytostomal orifice is encircled by a double row of trichites. The assembly of the oral structures is called "the cytopharyngeal complex". The body cilia are located on meridians, parallel to the longitudinal band of convexities of the pellicle. They are particularly distinctly seen in *Dileptus cygnus*. The posterior body end terminates as a caudal process bearing cilia as well. Contractile vacuoles occurring in a variable number are located on the dorsal body side.

The nuclear apparatus of *Dileptus cygnus* is constituted of macronucleus which is fragmented into over ten segments and of a variable number of micronuclei. In *Dileptus anser*, macronucleus consists of approx. 100 fragments suspended in cytoplasm. The number of micronuclei exceeds ten.

Ciliary movement and normal behaviour

The progressive movement of *Dileptus* is associated with the rotary one. The turn of the rotary movement as related to the progressive one, is not constant and may undergo changes. In forward advancing of the ciliate, bending of proboscic may be observed. It probably plays a locomotoric role. The observations of the ciliary movement in *Dileptus* (D o r o s z e w s k i 1963 a) were carried out after a previous slowing of the movement with methylcellulose or with low temperatures. The ciliary movement is metachronic. The ciliary waves are especially clearly observable on proboscis. In the cases observed, the ciliary waves run from anterior backwards, however this has not been proved as a general rule. The ciliary waves may be more or less distinct being characterized by their lability and variability. Just prior to reversal, a disarrangement of the metachronic waves cutline is observed. They efface and disappear. In the withdrawal phase of the cycle of the avoiding reaction, the course of ciliary waves was observed from the terminal body end forwards. After the conclusion of the reversal phase, the ciliate starts forwards at an altered angle. In many respects the behaviour of *Dileptus* at this cycle resembles to that of *Paramecium*.

Dileptus anser is a more motile ciliate than *D. cygnus* which leads a more sedentary mode of life.

In a resting position *Dileptus* lies on the bottom of the water container and performs oscillating movements with its proboscis. In the moment of contact of proboscis with the passing small ciliates, an outshot of trichocysts occurs. In the lying position the body cilia move very slowly and their waves are not observable on the body surface. In some cases, a full arrest of cilia was observable whereas the movements of proboscis occurred regularly. The course of the ciliary movement is not regular on the whole body surface. Zones of different kind and speed of movement may be distinguished. One of those zones is the cytostomal sector of proboscis with its feeding cilia.

This zone is distinguished by occurrence of very clear ciliary waves which may run from forward backwards or in the reversed direction. In feeding of the ciliate, the water current produced by those cilia cooperates in the transport of the killed prey towards the cytostome (D r a g e s c o 1962).

Another zone is that which encirles directly the cystostome. The cilia located on it move more slowly. A zone of a slow movement is also the caudal region. The remaining surface of the ciliate presents an integral unit with regard to the character of its movement. The most intense waves occur however on the anterior part of the ciliate as if they initiated the movement. It is not clear whether this initiation is performed by feeding cilia or by the remaining ciliature of proboscis.

Reactions to touching

As touch stimulation we understand the contact of the needle or another object with the ciliate body without evoking its impairment. The contact of a moving ciliate with an obstacle presents the same kind of stimulation. Essentially any sliding of a tool in water is accompanied by its movement and the side effect of water current is possible in similar way as in the case when

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the swimming ciliate reaches an obstacle. If the dimensions of the tool are small and its movement slow, the water currents produced by them may be neglected.

A series of experiments on touch stimulation of *Dileptus* was executed by means of micromanipulator of de Fonbrune (D o r o s z e w s k i 1963 b). Glass needles with special points were applied. The experiments proved that touching the cilia on proboscis or in the cytostome region evoked withdrawal i.e. a start backwards. Similar results had been reported by S e r a v i n 1962 in *Spirostomum* and in some other ciliates, among others in *Dileptus anser*. *Spirostomum* was also studied in this regard by C l a r k 1946. It was also stated in some cases that the reaction of forward start appeared after touching the posterior body end. For evoking this reaction not only the cilia should be stimulated but also the body surface of the ciliata. This was easily achieved with the highly motile ciliate as *Dileptus anser*.

Stimulation of the anterior part of a ciliate being in forward movement evokes reversal. During the backward movement, stimulation of the posterior part evokes forward movement. For ascertaining the interdependence of the reaction and the direction of stimulation, an experiment was made with sliding the needle along the body from the posterior end forwards touching only the cilia. In the moment when the needle has reached the cytostome area, withdrawal reaction takes place. This proves that the reaction depends on the place of the stimulus and not on the direction of its proceeding.

Experiments with the touch stimulation were also performed on fragments of *Dileptus anser* individuals after their transversal bisection (D o r o s z e w - s k i 1961). Stimulation of the anterior part of the fragments in forward movement evoked the reversal reaction, and a forward start — when the posterior parts in movement were touched. For these experiments only comparatively big fragments were used, as well the anterior as the posterior and middle ones.

Reactions to puncture

The ciliate studied was *Dileptus cygnus* as more appropriate for these experiments owing to its stationary mode of life.

Puncture with a sharpened glass needle was executed by means of the de Fonbrune micromanipulator (D o r o s z e w s k i 1963 b). In the resting individuals, puncture of the anterior body part evoked withdrawal, while puncting of the posterior one — a start forward. Puncture of the middle part of the body caused various effects. The boundary between both irritability runs accross the middle of the body.

A similar experiment was executed on ciliate fragments after bisection in the body middle. Puncture of the new-arisen anterior fragment evoked only withdrawal reactions, forward start was not ascertained in any case. In the posterior fragment the reactions were quite inverse: every puncture caused a forward start. The situation was altered after the onset of regeneration processes. Already after 45 min. following bisection, in the anterior part of the posterior fragment appears a sensitive area. Its stimulation caused withdrawal. A similar sensitive area appeared after 70 min. in the posterior part of the anterior fragment. Its stimulation involved a forward start. In both cases a changed shape of the fragment is marked under the influence of regeneration which proceeds very quickly in *Dileptus*.

Reactions to bisection

As another type of traumatic mechanical stimulus, the transversal bisection of the ciliate was performed. The direct motoric reaction was studied in both fragments obtained. Since a momentanous operation was essential, the micromanipulator was postponed and the section was executed manually by means of a sharpened steel needle.

The main experiments were carried out on *Dileptus cygnus* but some former experiments on *Dileptus anser* (D o r o s z e w s k i 1962) should be mentioned. Bisections were executed on the ciliate proceeding forward. A rapid guillotine movement (downwards) was performed. The arising posterior fragment showed an immediate reversal. The most posterior fragment showed no reversal continuing his forward movement.

A similar experiment was performed on *D. cygnus* (D o r o s z e w s k i 1965). The essential difference with *D. anser* is constituted by the fact that the ciliate was motionless in the moment of operation. Consequently its reaction was a backward start and not the usual reversal. Besides, a forward start was possible and this was much easier to ascertain than the reaction of acceleration of progressive movement in *Dileptus anser*.

If the section plane had run across the anterior part of the ciliate, both resulting fragments reacted with by withdrawal accompanied by rotation round the long body axis. After a bisection across the posterior region both fragments start forward. Bisection in the middle region may involve various reactions i.e. as well a progressive movement as a backward one in one or in both fragments. It the section has been repeated, it evokes a withdrawal ·in both new-arising parts in the anterior fragment. The result is inverse in the posterior fragment.

The regions delivering the reaction of forward and backward movement may be identified with the analogical regions determined for reactions to puncture.

Reactions to water shake

In the above experiments we had to do with mechanical stimuli acting locally. Different reactions were stated depending on the place of stimulus action. In the next experiments we tried to prove what would·be the reactions in the case when the mechanical stimulus has been applied to the whole cell of the ciliate i.e. when all the areas of reactivity are stimulated simultaneously. As such a stimulus the water shake was chosen. This stimulus has been investigated rather rarely. K i n a s t o w s k i 1962 studied the shrinkage of *Spirostomum* after a water shake. Besides, it was known that water shake may play a role in the onset of negative geotropism in *Paramecium*. It was proved by chance that a shake of Petri dish containing a culture of *Dileptus anser* evokes the reversal in nearly all the swimming ciliates, the individuals anchored to ·the bottom start swimming upwards and show a negative geotactic reaction.

We tried to follow this matter — in the first place — in *Dileptus anser* (D o r o s z e w s k i 1963 c). It was ascertained that the reaction to water shake may be not only the reversal but an acceleration of the forward movement as

well. However the movement acceleration is difficult to be defined and the cases of forward start are rare. For this reason the problem was studied when very prosperous cultures of *Dileptus cygnus* had been gained, with ciliates of a stationary mode of life. The reversal reaction was obtained in 90% of stimulated individuals. Later on, the ciliates were cut by half and in the posterior fragment, the reversal reaction appeared in 44% of stimulation cases, after the 1st hour — in 84%, and after the 2nd hour in 89%, i.e. as much as in the normal individual. This process is parallel to the course of the morphological regeneration. In the most posterior fragment, the corresponding values amount 1%, 17% and 52% respectively. This proves that at the initial phase, the posterior fragment was nearly deprived of any capability to reversal.

The subsequent experiments were performed on *D. cygnus* (D o r o s z e w - s k i 1968). Water shake was evoked by withdrawal of the clip of the microscope stage followed by releasing it. This method proved to be more simple and effective than producing the shake by means of a rod adjusted to electromagnet, as it was aplied previously. The ciliate experimented was placed on a concave slide in a drop of water surrounded by paraffine oil. The first series of stimulation was executed prior to operation and then about 15 shakes within 5 min. were applied at 1 hour intervals.

Prior to operation the ciliate may respond to the water shake by a forward start, a withdrawal or no reaction at all. The table visualizes the percentage of various reactions. After the ciliate has been bisected by half, the situation on the posterior fragment becomes changed essentially. Withdrawal fails to appear, start forward occurs in 99% and the lack of reaction only in 1%. Those reactions return gradually to the norm as seen in the Fig. 1. After 3 hours the
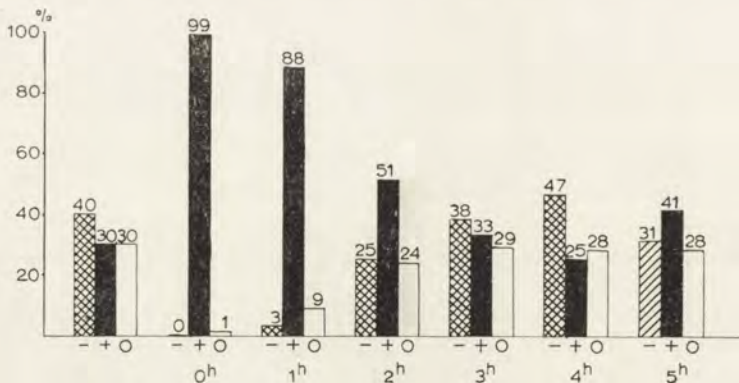
Fig. 1. Responses to the water shake in the posterior fragment of *Dileptus cygnus* (from D o r o s z e w s k i 1968) — withdrawal response, + forward start response, ○ no response

reactions are near the reactions in the normal individual. This occurs in the phase when the regeneration of the fragment has become much advanced and a certain area in its anterior part has been restituted. The reactions of the anterior fragment were also observed. An unexpected fact was ascertained that they are not analogical to the reactions reported above. In the anterior fragment, the only reaction to stimulation proved to be the withdrawal. This state keeps for over 5 hours.

Discussion

Now we try to construct the model of reactivity of *Dileptus* to mechanical stimuli on the base of the material presented by our experiments. "Stimulation of the anterior body part involves withdrawal, stimulation of the posterior one — a start forward". It is a striking fact repeated with an amazing regularity.

At the first glance it suggests an explanation that it presents an escape from the acting stimulus. A more intrinsic consideration however shows that it is not quite correct. The experiments with sliding the needle prove that the reaction depends on the action place of stimulus and not on the direction of its progressing. Besides, stimulation of the posterior end of the anterior fragment evokes withdrawal i.e. a movement towards the stimulus and not an escape of it. If it was an escape of the stimulus then after bisection, both halves would diverge, whereas this occurs only after a middle section. Bisection of the anterior part causes a backward movement in both new-arisen fragments and inversely.

It follows from the whole material gained from the experiments that we have to do with a cytological localization of reactivity since stimulation of certain defined regions involves a backward movement and of some others — a forward movement. This is associated with the axiality of organism to which corresponds the polarization of reactivity. Two zones of the *Dileptus* cell were distinguished: the "forward" and "backward response respond areas". The first occupies the anterior end of the ciliate and its stimulation evokes a withdrawal, the second area in the posterior part of the ciliate responds by a forward start to stimulation. Their boundary runs behind the line dividing the cell into two halves (excluding proboscis) (Fig. 2). It probably runs somewhat more backwards in *Dileptus anser* than in *D. cygnus*. A precise definition of its course meets difficulties because of absence of stable orientation points in this region. The above view is supported by the results of the experiments with stimulation by touching, puncture and bisection. The experiments are supported by the study of regenerating fragments. In these places where the morphological regeneration of the reactivity zones takes place — the reactions return also to the norm. The regularities described above occur in places where the local stimulation may be applied.

Another problem involves the reaction to water shake. This stimulus acts upon the whole cell, upon all its zones of reactivity. This supported by the results of experiments. Stimulation of the whole individual brings about as well withdrawal as forward start. Some stimuli remain without response. The percentage of those phenomena is similar which may be explained by alternate prevailing of different tendencies in the cell. Sometimes they annihilate each other and no reaction takes place. In the posterior fragment no cases of withdrawal are observed and start forward is the constant reaction. This coincides with the hypothesis of reactivity zones: according to it this fragment should be distinguished by the reaction of forward only. In the course of morphological regeneration of reactivity zones, the reaction returned to the norm.

Of course, this model of reactivity in *Dileptus* is too simple for being fully true. The specific receptor role of the cilia on proboscic and near the cytostome should be stressed. It was demonstrated — among others — in the experiments with touching. Proboscis is the cellular element which first meets the objects
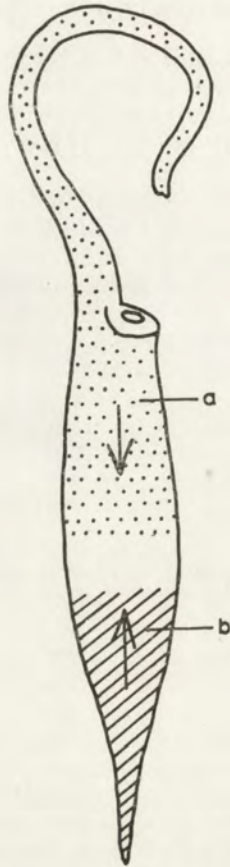
Fig. 2. *Dileptus cygnus.* (original)
a — backward response area, b —
forward response area

during the progressive movement of ciliate and this fact points out its possible receptive role. In this way, in the axial localization of reactivity, a specific role of buccal structures should be distinguished. Moreover in the case of *Dileptus*, the pattern of the oral structures is in conformity with the axiality of the body which is not the case in *Paramecium* (G r ę b e c k i 1965). In natural conditions, the action of reactivity zones may really be confined to the escape from the irritating mechanical stimulus. In the experimental conditions however more complex reactions come to light.

A certain asymmetry should be introduced to the model just described. Both response areas are not fully equivalent, the backward response area being in some sense more important. It is easier to evoke withdrawal by touch stimulation of the anterior body part than a forward start by stimulating the posterior one. Besides, the stability of withdrawal reaction persists longer on the anterior fragment in the course of regeneration than the stability of forward start on the posterior one.

In the experiments with the post-division individuals, proter showed only the reaction of withdrawal whereas in opisthe a differentiation of reactions occurred (D o r o s z e w s k i in press).

A similar phenomenon was noticed in the experiments with stimulation by water shake: on the anterior fragment persisted the withdrawal reaction only whereas the reactions of the posterior fragment have returned to the norm since a long time.

Ascertaining of the pattern of reception areas is in conformity with the theory of two opposite gradients in the ciliate body, as put forward by S e r a v i n 1962. This author did not restrict his study to mechanical stimuli. He ascertained that practically any fragment of *Spirostomum* may exhibit reversal under the action of a selected set of physical and chemical factors, although its susceptibility to those factors falls with the direction from front backwards.

The research of H o r t o n 1933 and A l v e r d e s 1922 demonstrated that any *Paramecium* fragment may react with reversal to chemical stimuli. It should be remarked that the results presented above concern only the reaction to mechanical stimuli studied in the buffer of Dryl or in Pringheim solution. In those media, the reproduction of *Dileptus* is normal. Their composition does not deviate much from the natural media.

In conclusion the reaction of forward start in *Dileptus* should be mentioned. We are inclined to consider the ciliate movement as continuous progressive forward movement, stopped at intervals by ciliary reversal. In the ciliate as *Dileptus cygnus* which is — as a rule — motionless, two reactions may take place: forward start and withdrawal. A l v e r d e s 1922 and D r y l 1952 ascertained that reversal is not the only motoric reaction of a ciliate. V e r v o r n 1897 revealed the acceleration of forward start after the action of water shake. S e r a v i n 1967 found the acceleration of movement in *Spirostomum* in many cases.

In the present communication, the analysis of behaviour of ciliate has been purposely restricted to 3 parameters: "+" — forward start, "−" — withdrawal, "O" — no reaction.

On the base of the above data an attempt was made to construct a simplified behaviour model of *Dileptus* in more or less normal conditions.

S u m m a r y

The results of investigations on the reactivity of *Dileptus* carried out in the course of several years, have been summarized. Touching, puncture and bisection were applied as stimuli. Water shake was also experimented as stimulus. The forward start reaction and withdrawal were studied in normal individuals as well as in the course of regeneration after bisection. It was ascertained that stimulation of the anterior body part evokes withdrawal and that of the posterior one — a forward start.

On the basis of those results, the existence of two reactivity areas in the cell of *Dileptus* was stated. One of them, situated on the posterior body part was determined as the "forward response area", the other one — on the anterior part of the ciliate — as the "backward response area".

STRESZCZENIE

Dokonano podsumowania wyników prac nad reaktywnością *Dileptus* prowadzonych w ciągu kilku lat. Stosowanymi bodźcami były dotknięcia, ukłucia i przecięcia. Wykonano również doświadczenia stosując jako bodziec wstrząs wody. Badano

reakcję ruszenia naprzód i cofania się na normalnym osobniku, oraz w przebiegu regeneracji po przecięciu. Stwierdzono, że podrażnienia przedniej części ciała wywołują ruch wsteczny, podrażnienia tylnej — ruch naprzód.

Ustalono istnienie dwóch stref reaktywności w komórce *Dileptus*. Jedna z nich jest położona na tyle ciała i podrażnienie jej wywołuje ruch ku przodowi, druga na przodzie ciała i podrażnienie jej powoduje ruch wsteczny.

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On the control mechanism of protoplasmic streamings in the plasmodia of *Myxomycetes*[1]

O mechaniźmie kontroli prądów protoplazmy w plazmodiach śluzowców

Plasmodia of acellular slime molds, which consist of relatively big masses of protoplasm organized in a network of channels, represent an exceptionally suitable material for the study of mechanisms of protoplasmic streamings. The main characteristic features of shuttle movements of the plasmodial endoplasm were rather exhaustively described by S e i f r i z 1943, S t e w a r t and S t e w a r t 1959 a, b, and by K a m i y a 1959. But the knowledge of protoplasmic streamings in the plasmodia is not only phenomenological. Due to intense study carried out with the plasmodia of slime molds a considerable piece of information has also been gathered about processes occurring in the plasmodial protoplasm and responsible for generation of the forces producing endoplasmic streamings. Among others it has been established that::

1. Endoplasmic streamings result from the gradients in the hydrostatic pressure along the plasmodial channels (K a m i y a et al. 1957). The forces responsible for generation of the gradients are released within the ectoplasmic gel (S t e w a r t 1964, J a h n 1964, J a h n and B o v e e 1964) or along the boundary between the ectoplasm and the flowing endoplasm (K a m i y a 1959).

2. The release of the forces producing protoplasmic streamings occurs during the processes of contraction of contractile proteins. The presence of contractile proteins corresponding to the actomyosin systems of vertebrate muscle tissues has been shown in the slime mold protoplasm by both methods, biochemical and electron-optical. L o e w y in 1952, T s'o et al. 1956, 1957 and N a k a j i m a 1964 isolated contractile proteins, myxomyosin and plasmodial myosin with ATP-ase activity; these perhaps represent the mechanochemical system involved directly in conversion of chemical energy (stored in ATP molecules) to mechanical one. W o h l f a r t h - B o t t e r m a n n (1963, 1964 a, b) demonstrated the identity of macromolecules, building up the fillaments

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[1] The paper when presented was illustrated with two 16 mm moving pictures: "The movements of plasmodia of the slime mold *Physarum nudum*", made by professor J. Z u r z y c k i and dr. L. R a k o c z y, and "Experimental modifications of protoplasmic streamings in the plasmodia of *Myxomycetes*", made by dr. L. R a k o c z y and dr. W. K o r o h o d a.

present in the plasmodial ectoplasm in its fibrillar structure with the biochemically isolated contractile proteins.

3. The processes of contraction are more intense in these regions of the plasmodia from which the endoplasm is actually flowing off than in those to which it is flowing in. The contraction is accompanied by heat production (Allen et al. 1963) and by differentation of fibrillar elements within the plasmodial ectoplasm (Wohlfarth-Bottermann 1964 a, b).

A majority of contemporary studies in this field concern the mechanisms of conversion of chemical energy into mechanical one but relatively little is known about the functions of the systems controlling the dynamic organization of the processes of the generation of forces producing protoplasmic streamings in the plasmodia. And it is question we should like to consider more broadly in the present report.

The characteristic, periodical changes in the direction and speed of the endoplasmic flow which can be observed in any part of the plasmodium suggest that there exist mechanisms operating in an oscillating manner. They seem to be distributed rather along the plasmodial network of channels than localized in any special region of plasmodium since the magnitude of the forces producing protoplasmic streamings was found to be independent of the length of plasmodial channels and the rhythms of the periodical reversals in flow are separate for individual channels, even if they are directly interconnected (Kamiya 1959). The analysis of the course of curves representing changes in the motive forces with time (so called dynamoplasmograms) was carried out by Japanese investigators. It showed that the plasmodia of slime molds represent polyrhythmic systems in which the forces measured result from the interference of forces generated during contractile processes occurring in numerous sites distributed along the plasmodium (Kamiya 1959). Such loci Jahn 1964 named "origins". They are supposed to operate separately and only additive effects of their activities are responsible for a general picture of changes in the protoplasmic streamings and in forces producing these streamings within slime mold plasmodia (cf. also Jahn and Bovee 1964). However Jahn similarly as Stewart or Kamiya did not explain what factors decide whether the particular region of plasmodial protoplasm (or rather ectoplasmic tube along the plasmodial channels) actually contracts or relaxes.

Perhaps one must assume that the phenomena of alternative contraction and relaxation processes in protoplasm at the last instance are controlled by local, periodically appearing differences in some metabolic activities of protoplasm itself. There is no other explanation of the autonomous character of contractile processes in plasmodial protoplasm. The forces are generated in plasmodia in an oscillating manner even if the plasmodia remain in constant external conditions. From that point of view the slime mold plasmodia correspond rather to big fresh water amoebae which actively produce pseudopodia even when suspended in fluid medium than to isolated cells of metazoa quickly rounding up and becoming immobile under such conditions. But though the last cause of the oscillations in the motive forces can be assumed to be associated with some metabolic reactions occurring also in an oscillating manner, it seems appropriate to look where within the plasmodial structures such reactions can take place and to search for their other manifestations than changes in the motive forces and in the endoplasmic flows.

Because of continuous gel-sol reactions accompanying the protoplasmic streamings the cytoplasmic structures are rather unstable. It makes it difficult to assume that the systems controlling the release of localized contraction-relaxation processes in ectoplasm work directly within the contractile protoplasm. It can be supposed that the relatively permanent structure characterized by a higher molecular organization than the cytoplasm would be more suitable to account for such functions. And, as a matter of fact a lot of undirect evidence suggests the localization of the systems controlling the release of contractile protoplasmic phenomena within the plasmalemma. The idea does not concern only slime molds but it is rather a general one. It was formulated as long ago as in 1906 and 1916 by L i l l i e who suggested that protoplasmic contraction wherever it takes place is always stimulated by eletrical depolarization of plasmalemma. At his time the suggestion was rejected since the plasmalemma was assumed by majority of authors to be absent in *Amoebae* and in slime molds where contraction takes place apparently (H y m a n 1917). But later on suggestions similar to that of L i l l i e have been repeatedly put forward. Direct evidence that cell membrane functions determine contractile reactions of internal cellular structures has been obtained for muscle and nervous tissue cells (cf. H o d g k i n and K a t z 1949, T o b i a s 1959 a, b, N a c h m a n s o n 1967, K e y n e s 1967). Similar phenomena have been postulated to occur in other cells too (cf. S e i f r i z 1953, S t e w a r t 1964) and recently some experimental evidences have been published which support such generalization (B i n g l e y et al. 1962, B i n g l e y and T h o m p s o n 1962, J e o n and B e l l 1965, N a c h m i a s 1964, 1968, B r a n d t and F r e e m a n 1967).

In plasmodia of slime molds the periodical changes in motive force orientation and magnitude were found to occur in an almost identical manner as the changes in eletrical potential difference measured between actually anterior and posterior regions of the plasmodia. The graphs representing both types of changes run parallelly (K a m i y a and A b e 1950). Suggestions that the electrical activities of plasmodia result from protoplasmic streamings (S e i f r i z 1943) could be safely rejected since the variations in potential differences continued in a characteristic fashion even in plasmodia strands consisting of not flowing, gelled protoplasm (K a m i y a and A b e 1950). But opposite relation possibility i.e. that the electrical activities of plasmodia reflect some functions of the mechanisms controlling the release of forces back of the streamings has never been sufficiently examined. This possibility was also excluded by K a m i y a and A b e 1950 as they observed the occurrence of some delay in the course of electric changes in relation to the changes in the motive forces as measured by means of the double-chamber method. K i s h i m o t o 1958 a, b, however, pointed out that it could be connected with the effects of pressure externally applied to plasmodium during measurements. Just recently L a y r a n d 1968 observed that any delay in phase disappears if the electrical changes are recorded simultaneously with changes in the direction of protoplasmic streamings but not motive forces. The same has been observed by us before the work of L a y r a n d had been published.

One can also point out that since there are so many analogies between the contractile phenomena in muscle tissue and in the plasmodial protoplasm it can be expected also that these processes are controlled by similar mechanisms in both materials. Changes in membrane permeability leading to translocation of

ions within the cytoplasm and exchange of ions between the protoplasm and the external medium may constitue, also in plasmodium, the triggering system for release of contractile cytoplasmic reactions. It is easier to measure the electric phenomena associated with the changes in ion distribution than the ionic fluxes themselves. Nevertheless the contractile processes in cytoplasm are stimulated rather by changes in the ratio of external and internal concentrations of K, Na, Cl, and Ca ions evoked by changes in membrane permeability then by the gross electric effects of these changes.

The above presented premises could lead to postulate the association of control system for generation of the forces producing protoplasmic streamings in plasmodia with the activities of plasma membranes, but the suggestion needed some experimental support. Perhaps the simplest way to show that the plasma membrane activities can stimulate cytoplasmic contractions is to inhibit such activities locally, in a restricted region of the plasmodium. It should produce local inhibition of contraction of the ectoplasm and thus, if the analogies with a contractile-hydraulic polytubular systems are valid. (S t e w a r t 1964, J a h n 1964) the inflow of the endoplasm into the affected regions of the plasmodium. Such experiments have been recently performed by us with the expected results (for methodical details of the experiments cf. K o r o h o d a et al. 1969). It has been known that the membrane activities can be reversibly affected by factors producing anaesthesia. Therefore to get desired alternations in the pattern of the protoplasmic streamings in plasmodia we had examined the action of several compounds known as anaesthetics on the plasmodia of two species, *Physarum nudum* and *Fuligo septica*.

Commonly used anaesthetics appeared unsuitable. Volatile general anaesthetics such as benzene, ether, chloroform etc. had to be excluded because they could not be applied locally in an exactly controlled manner. The local anaesthetics used contemporary in medicine have relatively big molecules what makes difficult their application to the plasmodia. The plasmalemma in this material is covered with a comparatively thick layer of a mucous coat and the diffusion of the active compounds to plasmalemma is delayed if possible at all. But the amide of benzoic acid which was used as an anaesthetic in XIX century (cf. H e i l b r u n 1943, p. 520) appeared to be the compound we had looked for. It is unvolatile, even in saturated solutions almost untoxic for living cells, and the shape, size and electric charge of its molecules suggested that it should be easily incorporated into the plasmalemma (for the discussion of conditions of chemicals incorporation into the lipoproteid membrane cf. W i l l m e r 1961). When the drop of the buffered solution of benzamide at concentration higher than 0.01 M (in diluted phosphate buffer or in 0.02 M solutions of NaCl or KCl) were externally applied onto seven days old plasmodia, rather dramatic changes in protoplasmic streamings took place [2]. First a shock reaction accompanied by slight narrowing of the plasmodial channels appeared but within 20 to 100 seconds the protoplasm started to flow into the affected region of plasmodium from all sides, through all channels adjacent to it. The influx of protoplasm into the region of plasmodium affected with the drop of benzamide solution lasted dozens of minutes (Fig. 1). For this time the reversals in the direction of protoplasmic flows were abolished in the channels directly leading toward affected areas. As a result in these regions

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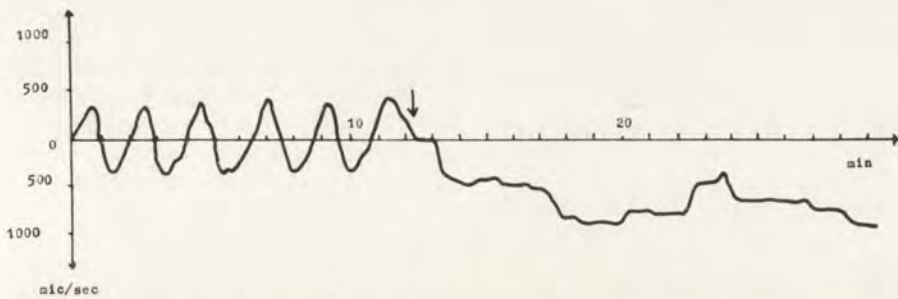[2] For the detail of the experimental procedure cf. K o r o h o d a et al. 1969.

Fig. 1. Changes in the speed and direction of protoplasmic streamings in the protoplasmic channel of plasmodium of *Physarum nudum* before and after application of a drop of benzamide solution. The moment of application is marked with an arrow. The changes in speed and direction of the streaming were recorded with the apparatus of Zurzycki (cf. Z u r z y c k i 1958)

of the plasmodium big masses of protoplasm accumulated and in some extreme cases even the whole plasmodium changed into a cluster composed of big drops of protoplasm. The accumulated protoplasm reached the volume up to 0.5 ml. It was not demaged permanently as when transferred onto a fresh medium it transformed within several hours into a normal plasmodium. The accumulation of protoplasm in the regions of plasmodia affected by application of the benzamide solution took part independently of the localization of those regions in plasmodia i.e. whether the solution was applied onto the frontal part of an advancing plasmodium or onto the posteriorly located network of plasmodial channels (Pl. I 1, 2).

The rhythmical reversals in the flows of protoplasm were abolished only in the channels directly leading to the affected areas. But even in the small branches of these channels the reversals continued and in the further located regions of plasmodia the pattern of protoplasmic streamings looked apparently normally. As shuttle streamings continued in unaffected parts of plasmodia, though the direction of protoplasmic flows in channels leading to the treated regions was constant, the speed changed reflecting probably the rhythms of streamings in the side channels.

The behaviour of accumulating protoplasm is interesting. It is immediately striking the eye that protoplasmic drops are always surrounded with a cell membrane and the endoplasm does not flow into the medium. The drops themselves have never had quite regular shapes. They are often cylindrical or resemble big plasmodial veins. The growth of accumulative masses of protoplasm occurs discontinuously. The drop of protoplasm never increases all dimensions simultaneously but the rather limited regions of its surface extend rapidly. The growth of that masses volume is extremely rapid and some time the rod-like structures about 10 mm in length and over 1 mm in diameter are formed within seconds. Rapid extensions of the drops appear to be separated by the periods during which the drop contraction occurs. Discontinuites regarding both, the distribution of extensions along the surface of the protoplasmic drops and the alternatively occurring contraction and extension processes in accumulating protoplasm seem to be associated with the membrane formation on the accumulated protoplasm. The benzamide molecules need some time before they can reach a newly formed surface of the plasmalemma by diffus-

ion and for this period of time the membrane can show perhaps normal activities. It can therefore trigger the contractions of ectoplasm which is also extremely rapidly formed in accumulative protoplasm. The endoplasm when flowing into the accumulative drops seems to be in a sol state but within drops it converts often rapidly into gel. When the endoplasm inflows into the drops it streams often smoothly but sometimes such rapid and chaotic movements within the protoplasm of the drops appear that they resemble the movements of water boiling in a pot.

One can assume that such a variety of flow patterns can be expected if the system controlling normally the sol-gel cytoplasmic reactions and the processes of protoplasmic contraction become unstable and disordered. And again it suggests that the cell membrane which is continuously formed de novo from the flowing protoplasm and simultaneously affected by the externally applied benzamide can fulfill such demands. The processes of the membrane formation during the described phenomena must be extremely rapid and effective. Unfortunately they are difficult to follow more precisely with light microscopes because of great dimensions of the accumulative protoplasmic drops. Only occasionally it was possible to note the phenomena corresponding to openings of the big endoplasmic vacuoles and an incorporation of their membrane into the plasmalemma but perhaps the membrane formation and growth can take place also on other ways (cf. K o r o h o d a et al. 1967).

The question whether the flow of endoplasm towards the affected with the benzamide solutions region of plasmodium is brought about by forces generated posteriorly to the flowing plasm or it is pulled up by forces of contraction within the accumulating protoplasm, can partly find an answer in the behaviour of the big vacuoles. In *Physarum nudum* plasmodia the big vacuoles with the diameter so great that they fill out completely the inner cross-section of channels are rather frequent. Since it is impossible to assume the transmission of the pulling forces through the vacuoles, one can conclude that the flow of endoplasm is brought about by the gradients of hydrostatic pressure resulting from the ectoplasmic rhythmical contractions in all regions of plasmodia except the affected one.

The observations of the effects produced by the local application of benzamide solutions upon plasmodia have been extended by following the electric activities of plasmodia (Fig. 2). Under normal conditions periodical changes of the electric polarization accompanying the reversals of the endoplasmic flow occurred in the manner similar to that described by K a m i y a and A b e 1950. Since the measurements and recordings have been fully automatized and it was possible to record the changes in electric potential differences for hours some new features in the electric activities of plasmodia could be also noted (K o r o h o d a et al. 1969). Potential difference oscillations characterized by long periods (in range of dozens of minutes) which had been suggested by K a m i y a and A b e could be recorded and they appeared to be associated with the direction of the plasmodium migration (Fig. 3). The general pattern of waves of the electrical changes appeared more complex when automatic recording was used than when the graphs were plotted on the base of succesive measurements. The application of a drop of the benzamide solution upon one part of the plasmodium and the one-directional flow of the endoplasm were accompanied by significant modifications in the electrical behaviour of the plasmodium. The effected part of plasmodium became relatively more nega-
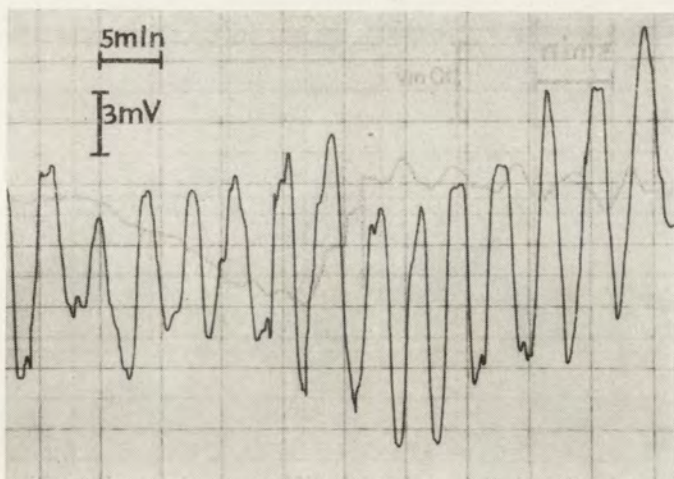
Fig. 2. Reproduction of an original recording of the periodical changes in electric potential difference as measured between two parts of plasmodial channel of *Physarum nudum* (K o r o h o d a et al. 1969)
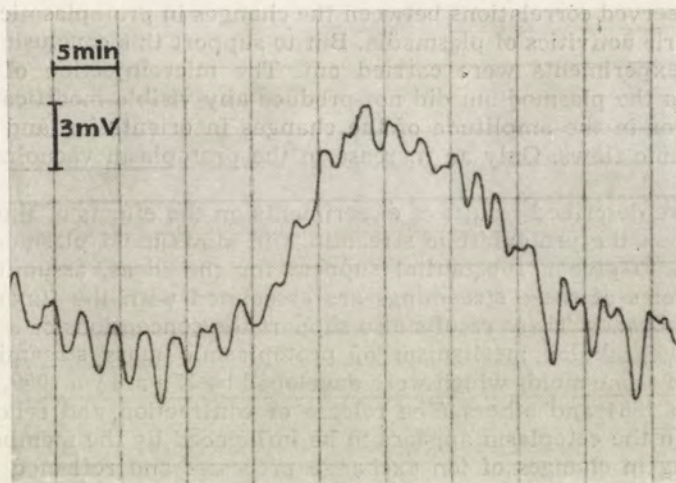

Fig. 3. Oscillations in the electric potential differences with short and long wavelengths

tively charged in relation to the unaffected one and the characteristic for slime molds periodical reversals in electric polarization disappeared (Fig. 4).

A close correlation has been observed between the character of the changes in protoplasmic streamings and electric potential differences along plasmodium.

The conclusion that the plasmalemma is a primary locus of the action of benzamide upon the plasmodia has been based mainly on premises stemming from an analysis of physicochemical properties of the benzamide molecules and the structure of lipoproteid membranes (G o l d a c r e 1952, W i l l m e r

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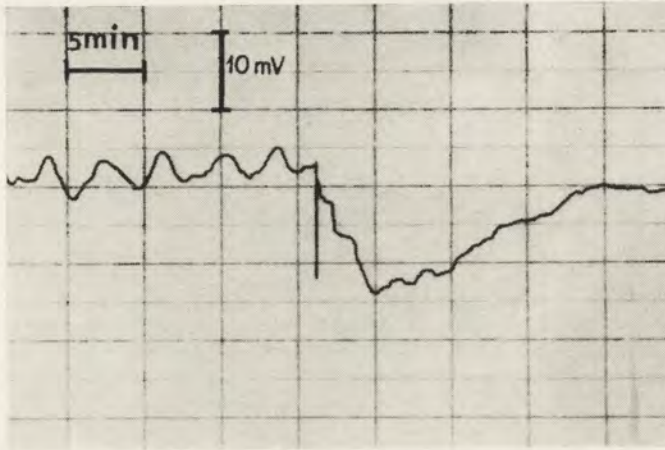
Fig. 4. Changes in the electric potential difference along plasmodium before and after application of benzamide solution (K o r o h o d a et al. 1969)

1961), from the observations of behaviour of an accumulating protoplasm and from the observed correlations between the changes in protoplasmic streamings and in electric activities of plasmodia. But to support this conclusion also some additional experiments were carried out. The microinjection of benzamide solution into the plasmodium did not produce any visible modifications in the periodicity or in the amplitude of the changes in orientation and in the rate of endoplasmic flows. Only an increase in the protoplasm vacuolization could be noted.

The above described results of experiments on the effects of the benzamide solutions upon the protoplasmic streamings in slime mold plasmodia seem in our opinion to give a substantial support for the ideas, assuming that the control systems of these streamings are associated with the functions of the plasma membranes. These results also support the conceptions of a contractile-hydraulic polytubular mechanism of protoplasmic mass streamings in the plasmodia of slime molds which were developed by K a m i y a 1959, S t e w a r t 1964, J a h n 1964 and others. The release of contraction and relaxation processes within the ectoplasm appears to be influenced by the membrane activities resulting in changes of ion exchange processes and reflected by changes in the electric activities of plasmodia. The continuation of normal flow reversals in the unaffected regions of plasmodium and simultaneous unindirectional flows of endoplasm into affected with the benzamide regions rather contradict the possibility of impulse transmission along plasmodial network of channels. This suggestion is supported also by K a m i y a's observations that the dynamoplasmograms are of composed character even when the forces in an individual channel are studied (K a m i y a 1959), T a s a k i and K a m i y a 1950 stated that the action potential appearing in the stimulated regions of plasmodium can not be transmitted along the plasmodial channels. Reverse results of B u r r 1955 were never confirmed by the other authors (cf. K o k i n a i J i g a d l o 1964) and also the present authors were unable to repeat them. The complex character of curves representing the potential difference changes accompanying the protoplasmic flow reversals in individual channels of plas-

modia was also recorded (cf. Fig. 2). It seems to suggest that the recorded potential difference changes stem from the interference of periodical, local electric potential differences appearing along the plasmodial channels. Two questions require further study: what is the mechanism of the periodical membrane activities operating as the triggering mechanism of contraction-relaxation and sol-gel protoplasmic reactions (1), and why these phenomena occur periodically even if there is no change in the external environment (2).

S u m m a r y

The premises leading to conclusion that the contraction-relaxation processes occurring in the cytoplasm of slime mold plasmodia and responsible for protoplasmic streamings are controlled by activities of cell membranes have been discussed. New experiments which confirmed this conclusion and indirectly also the contractile-hydraulic mechanisms of protoplasmic streamings in the plasmodia have been presented. It has been shown that the local anaesthesia produced by the benzamide results in an accumulation of big masses of living protoplasm in the affected regions of the plasmodium. The periodicity of reversals in direction and speed of protoplasmic streamings and in the electric potential differences along plasmodia was locally abolished by solutions of benzamide. Accumulation of the protoplasm was found to be accompanied by extremely rapid processes of the membrane formation and rapid, though often chaotic, sol-gel protoplasmic reactions.

STRESZCZENIE

Omówiono przesłanki sugerujące, że procesy kurczu i rozkurczu zachodzące w cytoplaźmie śluzowców i odpowiedzialne za prądy cyto-plazmy są kontrolowane przez zjawiska zachodzące w plazmalemmie. Przedstawiono nowe doświadczenia potwierdzające powyższy wniosek oraz wskazujące pośrednio na słuszność teorii tłumaczącej mechanizm powstawania prądów cytoplazmy w plazmodiach na drodze analogii z siecią kurczliwych naczyń. Wykazano, że lokalna anestezja wywołana przez amid kwasu benzoesowego powoduje nagromadzenie się protoplazmy w obszarach plazmodium traktowanych tym związkiem. Benzamid lokalnie znosił występujące w plazmodiach śluzowców rytmiczne zmiany w kierunku i szybkości prądów cytoplazmy oraz w elektrycznej polaryzacji plazmodium. Nagromadzeniu się proto-plazmy towarzyszyły zachodzące bardzo szybko procesy tworzenia błony plazmatycznej oraz żelifikacji protoplazmy.

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PLATE I
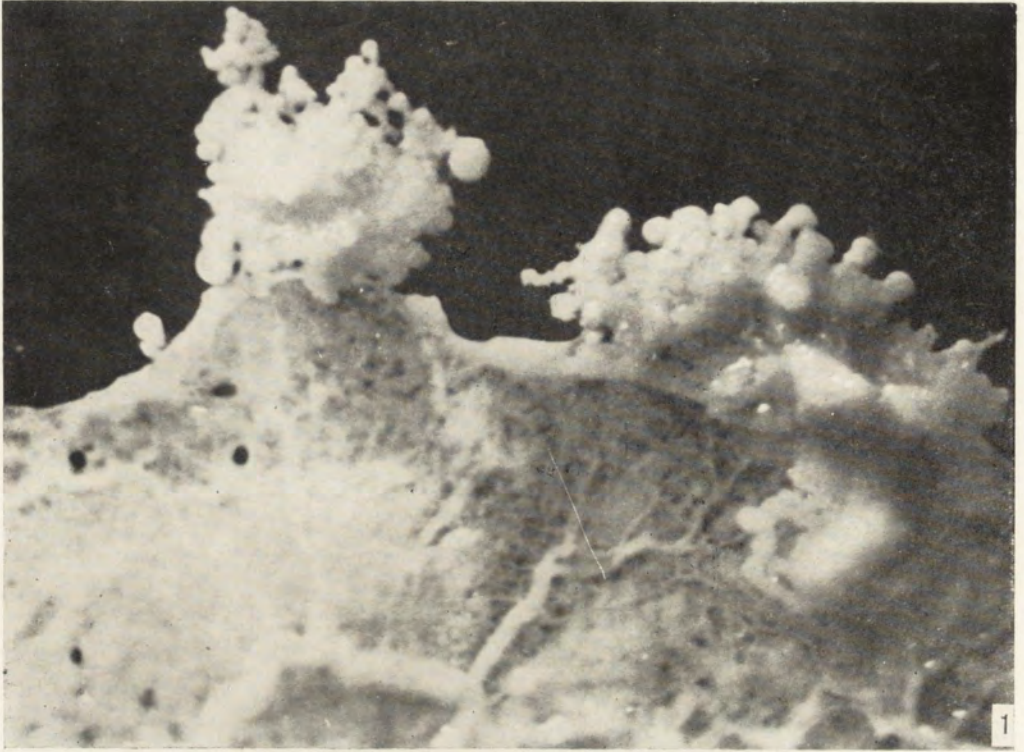
W. Korohoda et al. auctores phot.

W. KOROHODA and A. KUROWSKA

Quantitative estimations of the thresholds of electrotactic responses in *Amoeba proteus*

Ilościowe określenie progów reakcji elektrotaksji *Amoeba proteus*

Since 1896, when V e r w o r n had described the responses of an amoeba to direct electric currents (DC) the phenomenon has been frequently observed and discussed. The orientation of cell movements towards the cathode was observed in specimens of *Amoeba proteus* and *Chaos chaos* but not in *Amoeba dubia* (Mast 1931, D a n i e l and M a y 1950). Among those who tried to elucidate the mechanisms of electrotactic reactions in amoebae were C a l g r e n 1900, H i r s c h f e l d 1909, M c C l e n d o n 1911, Luce 1926, M a s t 1931, H a h n e r t 1932, H e i l b r u n n and D a u g h e r t y 1939, G o l d a c r e 1961, and 1964. They believed that: »the phenomenon may offer a clue as to the interpretation of the mechanism of amoeboid movement« (H e i l b r u n n 1943, p. 330). But the proposed explanations for electrotactic behaviour of amoebae were based on the general ideas of the amoeboid locomotion accepted actually by particular authors. If the surface pressure theories of amoeboid locomotion dominated, H i r s c h f e l d 1909 and M c C l e n d o n 1911 suggested that the DC decreases a surface pressure at the cathodal cell end. When so called sol-gel theories became generally accepted (cf. D e B r u y n 1947); L u c e 1926 and M a s t 1931 interpreted the cell responses as results of the influence of DC upon the sol-gel cytoplasmic conversions.

All interpretations of the responses of amoebae to the DC were based on the results of observations of cell behaviour. It is rather surprising but no investigations have been taken to characterize quantitatively the electric stimulus inducing responses in cellular behaviour and to determine the thresholds of electrotactic reactions in amoebae. The experiments presented in that report have been carried out to fill this gap.

Material and methods

Cultures of *Amoeba proteus* were grown on Chalkley's medium without calcium at 18°C. Before any experiment several hundreds of amoebae were washed three times with the solution used later as a medium in which observations and measurements were to be carried out. Then the animals were incubated in this medium for two to three hours and transferred into an experimental vessel. The vessel was made of perspex (Fig. 1). The reversible Ag/AgCl electrodes of relatively great surface area were mounted inside the electrode
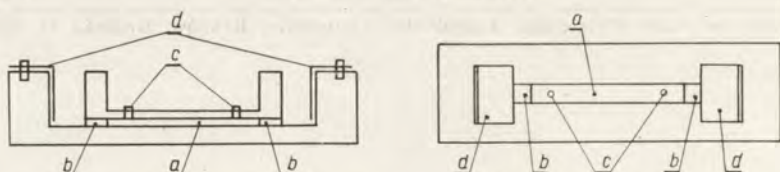
Fig. 1. Experimental vessel, vertical and horizontal cross-sections a — chamber containing amoebae, b — filters made of porous glass, c — cocks for an introduction of cells, d — Ag/AgCl electrodes

chambers and separated from the compartment containing animals by filters made of porous glass. The cross section area of the compartment containing amoebae was of 0.1 cm² (0.5×0.2 cm).

The observations of reactions of amoebae to DC were carried out in solutions of defined composition, buffered to pH 6.9 with the phosphate buffer solution (1 ml of 0.02 M PBS was added to 1000 ml of the solution). The electric conductivity of the solutions was measured with the conductivity meter, type 37—60, produced by »Eureka«. The stabilized DC generator (500 V, 250 mA) was used as a DC source. Desired intensity of DC passing through the experimental vessel was obtained by an adjustment of potentiometers connected in series into the circuit. The DC intensity was measured with MUR-4 mA meter.

Since the area of the cross section of the vessel (S), the DC intensity (I), and the electric conductivity of medium (X) were recorded during the experiments it was always possible to calculate density of DC passing through the medium containing amoebae $\left(\dfrac{I}{S}\right)$ and simultaneously the gradient of an electric potential i.e. the intensity of DC field in the medium surrounding animals $\left(\dfrac{I}{SX}\right)$ (cf. A b r a m s o n et al. 1942).

The observations of reactions of amoebae to DC were carried out with a microscope equipped with 20 and 40 × Cooke — A.E.I. long working objectives. Twenty animals had been placed into the experimental vessel and their reactions to given intensity of DC were followed within 15—30 seconds after the current had been switched on. Then new animals were transferred into the vessel and observed again at the higher DC intensity. The current was always switched on in such a manner that the electrode towards which an observed amoeba was originally moving became the anode. At any DC intensity the number of animals which did not reacted or reacted in one of three manners chosen as the index reactions was recorded. We called these reactions: weak electrotactic reaction, strong electrotactic reaction, and reaction of the membrane injury. They were distinguished by:

1. Weak reaction. After the DC has been started to flow through the medium, the movements of animals towards the anode got inhibited. Often the amoebae formed new pseudopodia oriented perpendicularly to the lines of electric forces, but if the current was switched off before 30 seconds passed, the original direction of cell movement usually reasumed.

2. Strong reaction. The DC stimulated the reversion in a direction of amoe-

bae movements by 180°. The wave of reversal of endoplasmic flow started at cathode, often at a close vicinity of the original uroid. The new tail was formed at the cell region which was originally a tip of an extending pseudopodium. The reversal of the cell movement direction appeared rather permanent. When the current was switched off within 30 seconds of its action it did not result in reasuming of the original direction of cell locomotion.

3. Reaction of the membrane injury. At relatively high intensities of DC the amoeba pellicle was broken at the anodal cell end and the cell contents were flowing out into the medium. The reaction took part exactly in the manner described by M a s t in 1931.

Experiments in any media were carried on as long as at some DC intensity all animals were killed. Then the tabulated results (cf. Table 1) were used for

Table 1

Reactions of *Amoeba proteus* to DC. Solution: 6 mM NaCl, pH 6.9, temp. 21°C

The stimulant			Number of animals showing			
DC intensity in mA	DC density in mAcm^{-2}	DC field intensity in Vcm^{-1}	lack of reaction	weak reaction	strong reaction	membrane injury
0.18	1.8	0.94	20	0	0	0
0.22	2.2	1.14	17	3	0	0
0.26	2.6	1.35	16	4	0	0
0.30	3.0	1.56	15	5	0	0
0.34	3.4	1.77	11	8	1	0
0.38	3.8	1.98	9	7	4	0
0.42	4.2	2.18	7	8	5	0
0.46	4.6	2.39	7	7	6	0
0.50	5.0	2.60	1	6	13	0
0.54	5.4	2.81	0	4	16	0
0.58	5.8	3.02	0	1	19	0
0.62	6.2	3.22	0	0	20	0
0.66	6.6	3.43	0	0	20	0
0.70	7.0	3.64	0	0	17	3
0.74	7.4	3.85	0	0	13	7
0.78	7.8	4.06	0	0	7	13
0.82	8.2	4.26	0	0	1	18
0.86	8.6	4.47	0	0	0	20

calculations of the DC densities and the DC fields which produced paticular responses in 50% of amoebae. The calculations were made according to the method of ED$_{50}$ estimation proposed by R e e d and M u e n c h 1938, which was described in detail by V e n u l e t and W ó j c i k 1960.

Results

The first, rather basic question which had to be solved was whether the electrotactic reactions in amoebae are determined by the intensity or density of the DC passing through the medium or by a gradient of the electric poten-

tial along the cells. To answer this question the series of observations was completed in solutions of NaCl buffered to pH 6.9 but of varies concentrations. The DC densities inducing the definite electrotactic reactions in 50% of amoebae were calculated separately for each concentration of sodium chloride. The results are presented in Fig. 2, and in Table 2. In Fig. 2 the ED_{50} of the reactions are presented as depending on the conductivity of solutions.

The obtained results showed clearly that the DC densities which stimulated particular electrotactic responses in amoebae depended almost linearly upon the electric conductivity of solutions. The higher was the electric conductivity of the solution used as a medium, the higher intensity and density of DC was
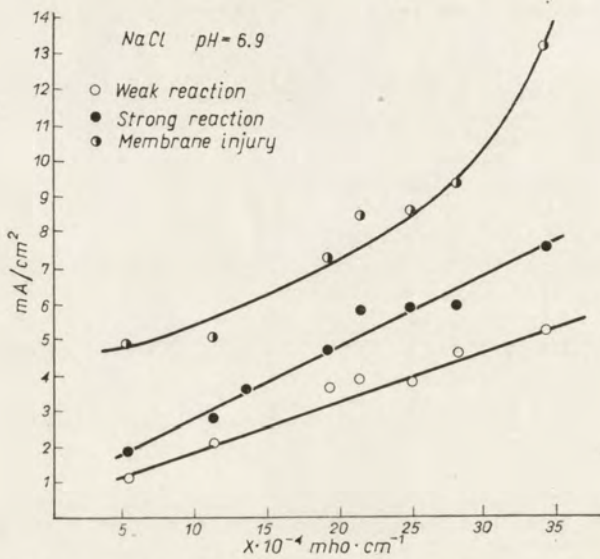
Fig. 2. ED_{50} of electrotactic reactions in *Amoeba proteus* as dependent on the conductivity of NaCl solutions used as the medium. The DC stimulating responses in amoebae defined by the current density given in mA cm^{-2}

Table 2

ED_{50} of electrotactic reactions in *Amoeba proteus* specimens, locomoting in buffered NaCl solutions. pH 6.9, temp. 21°C

Concentration of the solution in mM	Electric conductivity of the solution in ohm^{-1}cm$^{-1}\times10^{-4}$	DC density stimulating reaction in 50% of amoebae given in mAcm^{-2}		
		weak reaction	strong reaction	membrane injury
1.8	5.74	1.15	1.86	4.90
3.6	11.49	2.05	2.92	5.13
6.0	19.23	3.65	4.74	7.41
7.2	21.74	3.83	5.78	8.72
9.0	25.00	3.81	5.94	8.64
10.0	28.17	4.60	5.91	9.63
14.0	34.48	5.27	7.66	13.23

needed to produce the cell response. For example to produce the strong reaction in 50% of animals imbeded in 1.8 mM solution of NaCl, the current density 1.8 mA cm^{-2} was desired but to obtain the same effect in 9.0 mM solution of NaCl, the density 5.9 mA cm^{-2} was necessary.

The cell reactions appeared to depend much less on the medium conductivity when the DC was characterized by produced gradients in the electric potential, instead of the DC densities. The electrotactic reactions in amoebae were stimulated by nearly the same DC field intensity (i.e. the potential gradient given in V cm^{-1}) independently of NaCl concentration. Increase in the concentration of sodium ions produced rather slight decrease in the DC field required to stimulate the response in amoebae (Fig. 3 and Table 3).

The differences in the DC field intensities which produced the particular electrotactic responses in *Amoeba proteus* changed within 25% while the conductivity of medium was changed several times. Only in very diluted so-
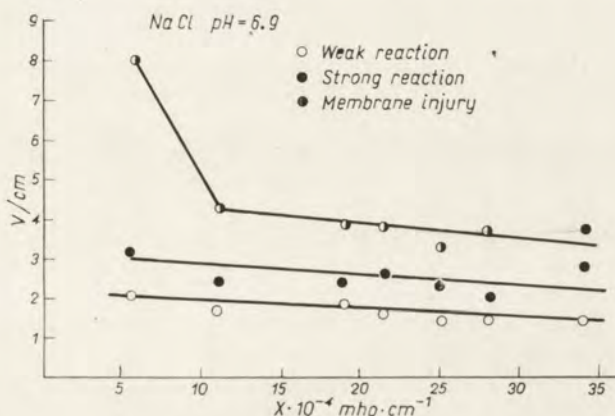
Fig. 3. ED$_{50}$ of electrotactic reactions in *Amoeba proteus* as dependent on the conductivity of NaCl solutions used as the medium. The DC stimulating responses in amoebae defined by the DC field intensity given in V cm^{-1}

Table 3

ED$_{50}$ of electrotactic reactions in *Amoeba proteus* specimens, locomoting in buffered NaCl solutions. pH 6.9, temp. 21°C

Concentration of the solution in mM	Electric conductivity of the solution in ohm^{-1}cm$^{-1}\times 10^{-4}$	DC field intensity stimulating reaction in 50% of amoebae given in Vcm^{-2}		
		weak reaction	strong reaction	membrane injury
1.8	5.74	2.0	3.2	8.1
3.6	11.49	1.8	2.5	4.4
6.0	19.23	1.9	2.5	3.9
7.2	21.74	1.7	2.6	4.0
9.0	25.00	1.5	2.4	3.5
10.0	28.17	1.5	2.1	3.9
14.0	34.48	1.5	2.9	3.8

lutions the reaction of membrane injury was induced by DC field of relatively high intensity. One could suppose that it resulted from the slower exchange of traces of calcium from the amoeba membrane in diluted solution. The traces of calcium could well appear in the culture medium because of wheat grains being there. To test this suggestion one series of measurements was made in the medium composed of 4 mM of NaCl and 1 mM of $CaCl_2$. It was found that the ED_{50} of cell responses given in the DC field intensities were under such conditions 2.6, 3.2, and 13.5 V cm^{-1} correspondingly. The thresholds of the weak and strong reactions increased therefore about 30% when compared to that in pure NaCl solution. The membrane injury reaction however occurred only in the fields more than three times stronger than those producing indentical reaction in amoebae imbeded in a medium without calcium.

It was discussed by several authors if the reversal in a direction of amoeba locomotion exposed to the DC resulted primarily from processes of a stimulation of pseudopodia extension towards the cathode (L u c e 1926, M a s t 1931) or from the inhibition of pseudopodia formation directed to the anode (G o l d a c r e 1961, 1964). Since the wave of reversal in a direction of the intracellular endoplasmic flow started always at the cathode one could assume rightness of the first alternative. But some observations lead to opposite conclusion. When amoebae, just contracted after strong mechanical stimulation, were exposed to DC of high intensity, the pseudopodia formation at the cathodal cell end could be never induced. The reaction of membrane injury took place normally as in locomoting cells. The same was found when animals did not move, displaying pinocytosis. The pinocystosis was induced by addition of 1% of bovine plasma albumins (C h a p m a n - A n d r e s e n 1962). These observations seem to support rather the oppinion of G o l d a c r e. His suggestion that the protoplasmic contraction is stimulated by the DC within anodal cell regions seems to be also in agreement with the results of A l s u p 1939 on reactions of amoebae exposed to alternating electric currents.

Discussion

The presented results univocally indicate that the electrotactic responses in *Amoeba proteus* are dependent upon the gradients of electric potential i.e. upon the DC field intensity in the medium and not upon the intensity and density of the DC passing through the amoeba environment. It shows that the DC fields act primarily upon the cell structures and their action is not intermediated by the ionic flows passing through the medium. One can also suggest on the base of observations of amoeba responses that there is not all or none response in animals exposed to DC fields. If it was so, the differences between the weak and strong reactions would be absent. On the other hand when the DC field intensity is high enough to reverse permanently the direction of amoeba locomotion, its further increase has no effect on the speed of cell movements. It indicates that the externally applied electric fields do not provide the forces and energy directly responsible for cell movements but only stimulate and orient the forces generated by cells themselves. Similarly as it was earlier observed by A n d e r s o n 1951 in slime mold plasmodia, also in *Amoeba proteus* the speeds of cell locomotion and endoplasmic streamings do not depend neither upon the DC density nor upon the DC field and potential

gradient along cells. It is shown that the suggestions of C a l g r e n 1900 and later H e i l b r u n n and D a u g h e r t y 1939 (cf. also H e i l b r u n n 1943, and 1956) who supposed the electrotactic responses in amoebae to be brought about by the protoplasmic streamings resulting from electrokinetic phenomena are wrong. These suggestions can be safely rejected as no one can postulate too, that the DC fields of intensities 2 to 4 V cm^{-1} could produce electrophoretic or electroosmotic flow of endoplasm at the rate 20—40 mic sec^{-1} (cf. A b r a m s o n et al. 1942 for discussion of electrophoretic mobilities of proteins). And such rates of the endoplasmic streamings are often found in amoeba pseudopodia (A l l e n 1961).

The quantitative methods used in our experiments may be useful for further study of the effects of various factors on cell electrotactic reactions. Their application showed that for any study of the electrotaxion of amoebae the description of the stimulus by measurements carried out only for the DC intensity and density is unsufficient. The electric potential gradient i.e. the intensity of the electric field acting on cells and inducing their responses depends also upon the medium conductivity. It is therefore impossible to repeat quantitatively any of experiments on electrotactic reactions of amoebae in which only the DC density was determined. Perhaps it is also true for studies on electrotaxis of other cell types. One can note too, that for quantitative observations only the reversible electrodes can be safely used for DC input to fluid media. When polarizeable electrodes are used, the cells respond to the stimulus which changes its intensity with time in an uncontrolled manner (cf. A b r a m s o n et al. 1942).

The experiments carried out in the medium enriched in calcium ions indicated that under such conditions the thresholds of the electrotactic reactions are slightly increased but the cell membrane injury is caused only by relatively strong DC fields. It proves the importance of calcium in the maintenance of the cell membrane stability. The presence of calcium ions in the cell medium makes the observation of the electrotaxis of amoebae relatively safe, as the injury in amoeba pellicle can be easily avoided.

Summary

The ED$_{50}$ of electrotactic reactions in *Amoeba proteus* were determined. It was found that the cell responses depended upon the intensity of the DC field effecting the cells but not upon the ionic current passing through the medium. The consequences of it have been discussed.

STRESZCZENIE

Wyznaczono ED$_{50}$ reakcji elektrotaksji *Amoeba proteus*. Stwierdzono, że reakcje komórek są określane przez natężenie pola elektrycznego działającego na komórki a nie przez prąd jonowy płynący przez środowisko. W dyskusji omówiono znaczenie tego stwierdzenia.

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PROTISTOLOGICA

Fasciculi praeparati:

Z. R a a b e: Ordo *Thigmotricha* (*Ciliata* — *Holotricha*) III. Familiae *Ancistrocomidae* et *Sphenophryidae* — P. C. B r a d b u r y: *Urceolaria kozloffi* sp. n., a symbiont of *Brachiopods* [*Urceolaria kozloffi* sp. n., un symbiont des brachiopodes] — И. В. Б у р к о в с к и: Инфузории месопсаммона Кандилакшского залива (Белое море) I. [The Ciliates of the Mesopsammon of the Kandalaksha Gulf (White sea) I] — P. R. E a r l: Some protozoan endosymbionts in Ohio-frogs [Quelques protozoaires des grenouilles en Ohio] — M. J e r k a - D z i a d o s z: Studies on the distribution of trichocysts in the normal life cycle and during regeneration of *Urostyla cristata* Jerka-Dziadosz, 1964 (*Hypotricha*) [Badania nad rozmieszczeniem trichocyst w normalnym cyklu życiowym i podczas regeneracji *Urostyla cristata* Jerka-Dziadosz, 1964 (*Hypotricha*)] — R. W. A s h f o r d: Some relationships between the Red Flour Beetle. *Tribolium castaneum* (Herbst) (*Coleoptera, Tenebrionidae*) and *Lymphotropha tribolii* Ashford (*Neogregarinida, Schizocystidae*) [Observations sur les relations entre la néogrégarine *Lymphotropha tribolii* Ashford et le coléoptère ténébrionide, *Tribolium castaneum* (Herbst)] — H. M a c h e m e r: Primäre und induzierte Bewegungsstadien bei Osmiumsäurefixierung vorwärtsschwimmender *Paramecien* [Primary and induced stages of movement after osmium-instantaneous fixation of forward swimming *Paramecium*]

Fasciculi: