ACTA PROTOZOOLOGICA

VOLUMEN XI

SYMPOSIUM

MOTILE SYSTEMS OF CELLS

WARSZAWA 1972

This special volume of Acta Protozoologica contains the papers presented during the International Symposium "Motile Systems of Cells" held in Cracow between August 3rd and 7th, 1971. This Symposium was organized as a result of joint efforts of M. Nencki Institute of Experimental Biology, Polish Academy of Sciences in Warsaw and the Institute of Molecular Biology, Jagiellonian University in Cracow.

It was the intention of the organizers of Symposium to cover the problem of cell motility within the broad scope with special attention paid to: molecular basis of cell motility and ultrastructure of cell motor organelles, motile systems in amoebas, myxomycetes and tissue cells, intracellular movements, the mechanism of the flagellar and ciliary movements, excitability and motor response to external stimuli (taxes kineses etc.). In this way the assays from this volume of Acta Protozoologica provide an illustration of various kinds of experimental approach to the problem of cell motility with special emphasis placed on the primary biological systems with exclusion of contractile systems in muscle cells, which are the subject of highly specialized separate meetings. It should be pointed out that more than 50 participants from twelve countries took part in the Symposium, which included 8 separate sessions including the final closing session devoted to the general discussion.

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MOTILE SYSTEMS OF CELLS

KRAKÓW, AUGUST 3–7, 1971

REDACTORES

S. DRYL et J. ZURZYCKI

WARSZAWA 1972

POLISH ACADEMY OF SCIENCES

M. NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

ACTA PROTOZOOLOGICA

SPECIAL VOLUME, DEVOTED TO INTERNATIONAL SYMPOSIUM
"MOTILE SYSTEMS OF CELLS" KRAKÓW AUGUST 3–7, 1971

Scientific editors of volume
S. DRYL and J. ZURZYCKI

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INTRODUCTION

This special volume of Acta Protozoologica contains the papers presented during the International Symposium "Motile Systems of Cells" held in Cracow between August 3rd and 7th, 1971. The Symposium was organized as a result of joint efforts of M. Nencki Institute of Experimental Biology, Polish Academy of Sciences in Warsaw and the Institute of Molecular Biology, Jagiellonian University in Cracow.

The idea of compiling a collection of essays on various aspects of cell motility in the animal and plant kingdom originated during discussions at the Symposium "Physiology of Motor Response in Protozoa", organized during cellebrations of 50th Anniversary of M. Nencki Institute of Experimental Biology, December 9–12, 1968. One of the editors of this volume wrote in 1970 to nearly 100 cell motility specialists from various countries in the world, asking about their possible contributions to the planned meeting. This met with an extremely enthusiastic response which reflected a high interest of cell biologists from all over the world in this particular field of research.

It was the intention of the organizers of Symposium to cover the problem of cell motility within the broad scope with special attention paid to: molecular basis of cell motility and ultrastructure of cell motor organelles, motile systems in amoebas, myxomycetes and tissue cells, intracellular movements, the mechanism of the flagellar and ciliary movements, excitability and motor response to external stimuli (taxes kineses etc.). In this way the assays from this volume of Acta Protozoologica provide an illustration of various kinds of experimental approach to the problem of cell motility with special emphasis placed on the primary biological systems with exclusion of contractile systems in muscle cells, which are the subject of highly specialized separate meetings. It should be pointed out that more than 50 participants from twelve countries took part in the Symposium, which included 8 separate sessions including the final closing session devoted to the general discussion. The papers are presented in the same order as they were read at Symposium, the short lasting discussions after each paper and the general discussion held during the last day of the meeting had an informal character and for that reasons they are not included in the material of Symposium. The references at the end of papers may serve as an effective guide to the literature.

The organizers of Symposium want to express sincere thanks to all the participants of Symposium for their active contribution both in the exchange of views during the meeting and in the supply of the manuscripts for their final publication in the present volume. The organizers hope that the published papers of Symposium "Motile Systems of Cells" will provide an important information concerning the recent advances in various aspects of cell motility studies. Let us believe that this volume will prove to be a valuable book for the cell motility students and will have both useful and stimulating effects on the further progress in this particular field of experimentation.

We wish also to express our appreciation to the Polish Academy of Sciences for support which enabled us to organize the Symposium. Our separate thanks are extended to the Vice-President of Polish Academy of Sciences prof. M. Mięsowicz for his kind and valuable remarks during closing Session and to the Administrative Director of Cracow Division of Polish Academy of Science Mr. Janusz Wiltowski and Mrs. F. Skolimowska for their contribution to the ogranization of Symposium.

STANISŁAW DRYL et JAN ZURZYCKI

February 1972 Organizers of Symposium "Motile Systems of Cells'

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KRAKÓW, AUGUST 3–7, 1971

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Cell shapes and cell contacts

Morphogenesis during normal development, in tissue regeneration and in the growth of neoplasia in *Metazoa* involves changes in the coordination of relative rates of growth, cellular interactions and cellular migrations. In addition, the stable shape and ultrastructure of the individual cells forming the tissues plays an important role. The role of cell adhesion, cell motility and ultrastructure in determining cell morphology and the type of contacts which cells can make with each other and with other tissues will be the subject of this paper. Recent work by a number of authors, using thin sections in the electron microscope (Ishikawa, Bischoff and Holtzer (1969), and Bhisey and Freed (1971)) has shown in more detail the importance of cytoplasmic microfibrils and microtubules in relation to cell function. As the stereoscan electron microscope, in combination with ion etching of the surface (Ambrose, Osborn and Stuart 1970, Ambrose, Batzdorf, Osborn and Stuart 1970) provides a more general three-dimensional view of the cell and its ultra structure, this method has been used in the present investigation. This has been accompanied by parallel work on the living cells using time lapse cinematography.

Experimental methods

Stereoscan microscopy

Cultured specimens on glass coverslips were fixed in two ways:

1) Freeze substitution. After washing with protein-free synthetic culture medium at 37°C to obtain a clean cell surface, the specimen was plunged immediately into a tube of liquid propane in liquid nitrogen. It was then transferred to a tube of absolute alcohol in dry ice. Alcohol was allowed to replace water overnight. The specimen was allowed to warm up slowly at room temperature and dried in air. It was then coated with gold palladium for microscopy.

(2) Specimens were first fixed with glutaraldehyde as for E. M. sections and subsequently subjected to freeze substitution.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Ion etching

The cell surface was etched by bombardment with hydrogen ions (Ambrose, Osborn, and Stuart 1970) and subsequently coated with gold palladium for stereoscan microscopy.

Time lapse cinematography

Standard Wild time lapse equipment was used in combination with a Smith–Baker interference microscope. Interference and Reichart Anopteral phase contrast objectives and condensers could be interchanged on slides.

The surface contact microscope (Ambrose 1956) was also used for cine work with a high pressure and high intensity mercury arc.

Adhesion of cells to substrate

The problem of the adhesion of cells to glass and other surfaces has been investigated by a number of workers. Also the nature of adhesive forces between cell surfaces, the role of the negatively charged surface in producing a repulsive force, of sialic acid residues in contributing to the negative charge, the role of calcium ion in forming bridges between groups on the surface and between surfaces etc., have been described elsewhere (Ambrose 1967). Figure 3 indicates the type of interaction between charged groups and Ca^{++} ions which may be involved.

In this paper we shall only concern ourselves with cell surface adhesions in so far as they involve cell surface topography.

Cell shapes and cell movements

When tissue cells are isolated by the use of trypsin, EDTA, collagenase or other agents and suspended in medium at low temperature, almost all of them assume a spherical shape. A sphere is the shape which has the minimum cell surface to volume ratio and, hence, the minimum surface energy. It is likely that minimum surface energy is the condition which determines the shape of such cells, although it is known that the surface tension is very low.

When the temperature is raised an extremely active movement of the plasma membrane begins to take place. The main activity is blebbing, due to expansion of large rounded pseudopodia which suddenly spring back to the original surface contour (Fig. 1a). This behaviour is similar to the appearance of cells in mitosis.

Even after cells have begun to attach to a solid substrate this blebbing activity persists in those parts of the cell not in contact with the substrate. This activity probably represents some surface instability because lymphocytes, which normally spend part of their life in suspension in the peripheral blood, do not produce this type of behaviour. They tend to produce fine pseudopodia (millipodia) which probably

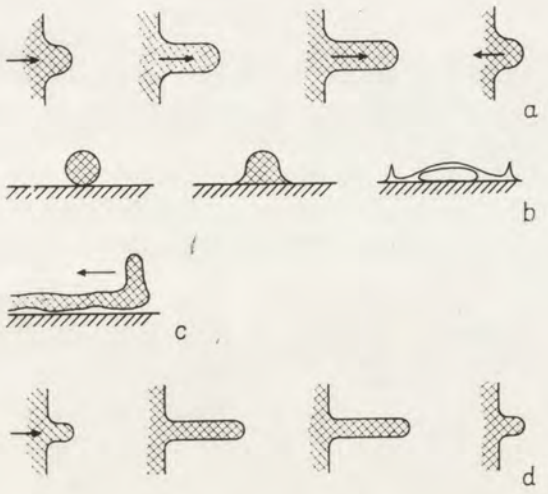

Fig. 1. a — stages in the building up and retraction of a large bleb on the cell surface, b — stages in the spreading of a cell settling from suspension on a glass surface, c — large lamellipodium on the outer border of a spreading cell, d — stages in the extension and retraction of microvillae. These filaments contain microfibrils lying parallel to the axis

account for the dancing type of locomotion shown by these cells when they settle on glass (Plate I 1); once settled on the glass, lymphocytes retain their somewhat rounded form but with a hand mirror appearance due to the formation of a stable tail; they probably make contact with the solid surface only by means of the millipodia shown in Plate I 1. But with normal mammalian fibroblasts, ectodermal and other tissue cells there is a progressive spreading of the cell which is accompanied by intensive ruffling of the cell membrane on the leading edge. An undulating type of movement is known to occur also on the surface in contact with the glass and provides the main locomotory mechanism for these cells by generating intermittent contacts between the cell surface and the glass surface (Ambrose 1961, Korohoda 1970). The undulating movements of the membrane will be considered in more detail later. The surface topography in an undulating region on the upper surface is shown in Plate I 2. The process of cell spreading continues progressively until the cell is, in most cases, brought into the form of an extremely thin sheet of protoplasm (a few hundred Å thick at the edges) with greater thickness over the nuclear area. At the same time microvillae (rather rigid pseudopodia of uniform diameter of 2000–3000 Å) form both on the upper and lower surface of the cell (Fig. 1c and Plate I 3). They can be seen attached to the glass surface in Plate I 4.

This spreading process is evidently partly connected with adhesiveness to glass and partly with dynamic activity of the cell surface complex consisting of cell surface coat, lipid membrane and subsurface structures (Ambrose and Easty 1970).

The shape of cells

Rapid spreading of the kind described above can lead to the formation of disc-like cells; a number of cells of ectodermal and endodermal origin almost assume such a shape on glass, although there is usually some slight polarity produced leading to the appearance of a tail region (Fig. 2a). But normal fibroblasts rapidly begin to show polarisation of their movements; they assume a fan–like sheet with a broad ruffled membrane on the leading edge (Fig. 2b). Sometimes there are two or more fan-like

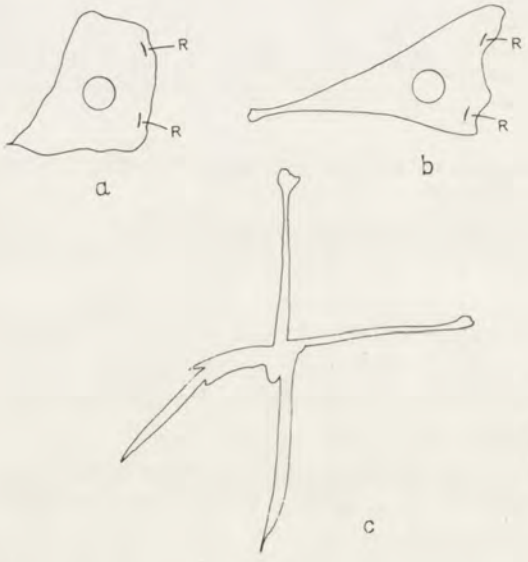

Fig. 2. a — shape of a spread cell on smooth glass of ectodermal or endodermal origin, b — shape of a fibroblast spreading on smooth glass, c — star-shaped pseudopodia of an astrocyte, R — Region of ruffled membranes

pseudopodia in which one is dominant in controlling movement for a limited period. As the culture grows and approaches confluence, contact inhibition of cell movements, due to side contact, leads to increased elongation of the cells which become extremely elongated with one leading fan-like pseudopodium (Fig. 3 d).

The other cell types we have studied in detail are astrocytes, which form the connective tissue of the brain (Ambrose, Batzdorf and Easty in press). They show a quite different morphology. They tend to spread in the form of extremely extended and radiating pseudopodia, with branches. Fan-like pseudopodia are seen at the ends of these long processes.

The main experimental work to be described below has been an attempt to explain, in term of adhesiveness, cell movements and internal ultrastructure, the difference between the morphology of fibroblasts and astrocytes as seen on monolayer culture.

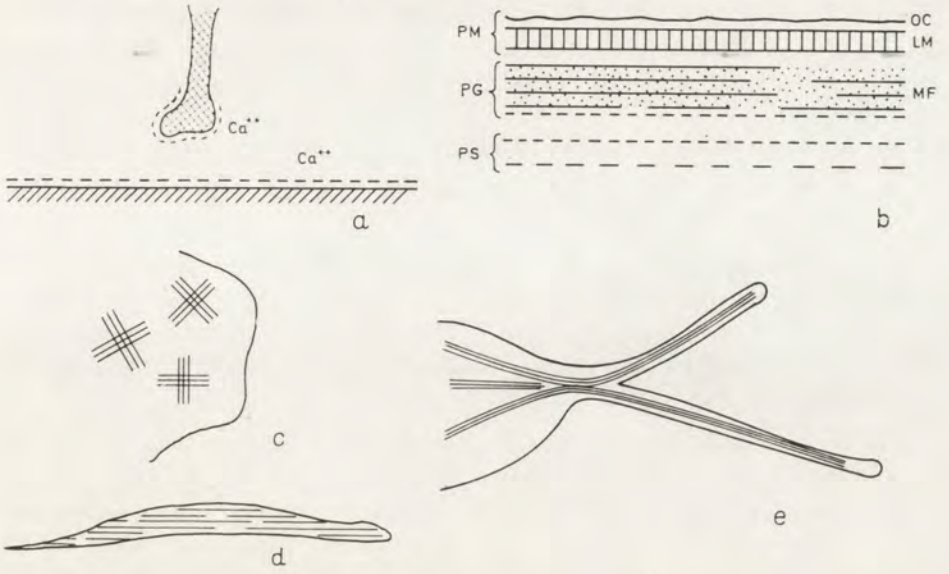

Fig. 3. a — fine pseudopodium approaching a plane surface. The net charge density is reduced in regions of high curvature. Ca^{++} ions are able to form bridges between appropriately spaced groups carrying negative charge, b — generalised structure of the cell surface complex. PM: plasma membrane. OC: outer coat. LM: lipid membrane. PG: plasma gel MF: 60 Å microfibrils. PS: plasma sol, c — molecular felt of microfibrils sometimes seen in disc-shaped cells after ion etching, d — highly orientated layer of microfibrils seen in elongated fibroblasts in a confluent layer, e — 5000 Å diameter bundles of microfibrils seen in glial cells.

Sub-surface structures

(a) Ion etching studies (Ambrose, Osborn and Stuart 1970, Ambrose, Batzdorf and Easty in press) indicate that the cell surface complex of tissue cells is organized somewhat as shown in Fig. 2b). On the outer surface lies a coat consisting partly of glycoproteins. Below this is found the lipid phase or plasma membrane. Beneath this again lies the plasma gel. This is variable in thickness. It may be only a few hundred Å thick in some tissue cells whereas in plasmodia it may be several microns thick. Embedded in this gel lie microfibrils, orientated parallel to the plasma membrane. Ion etching shows that these can be revealed by a critical amount of etching of the surface. With light etching, patches of plasma membrane can still be seen on the surface, with areas of plasma gel exposed. No fibrils can be seen at this stage. Heavy etching removes the fibrous region completely. In Pl. II 7 most regions have been too heavily etched and have penetrated into the cell matrix. In one area subsurface fibrils can be seen. With a critical level of etching some cells exhibit an extremely regular assembly of sub-surface fibrils (Plate II 8). Once the cell culture has

grown to produce a layer of confluent fibroblasts the cells become extremely elongated (Fig. 3d). A uniform layer of fibrils may be seen which are orientated along the length of the cell and are parallel in adjacent cells. In low density cultures both fibroblasts and epithelial cells tend to show only patches of orientated fibrils and many regions resembling a molecular felt (Fig. 3c).

Astrocytes, on the other hand, tend to show orientation of subsurface fibrils at all stages of culture growth.

These can even be seen in attentuated regions without ion etching. Even in the cell body such fibrils are found (Plate II 10). They are, in this case, arranged in bundles of about 5000 Å diameter. There are indications that these bundles may branch in the regions where the star-shaped pseudopodia characteristic of these cells are produced (Figs 2c and 3c).

(b) The cytoskeleton. The evidence described above suggests that the organisation of the subsurface fibrils can be correlated to some extent with the type of cellular morphology; a molecular felt being seen in flat cells, sheets of fibres in elongated cells, and fibrillar aggregates in cells which produce branching pseudopodia. Further evidence in favour of this view is provided by a study of malignant cells. In BHK 21 fibroblasts, after transformation with polyoma virus, the cells develop more numerous elongated pseudopodia than the untransformed cells; they lose contact control of mitosis and contact inhibition of movement is less marked than in untransformed cells. The organisation of microfibrils, seen after ion etching, in the most extended pseudopodia, is reduced in comparison with untransformed cells. In human astrocytes there is a progressive decrease in the capacity to form extended starshaped pseudopodia with increasing malignancy. Grade IV astrocytomas have merely truncated and irregular pseudopodia; organised subsurface fibrils are completely absent (Ambrose, Batzdorf and Easty in press).

The minimum size of sub-surface fibrils, revealed by ion etching and stereoscan microscopy, is ~300 Å. This is near the limit of resolving power. Thin sections reveal that the microfibrils embedded in the plasma gel of fibroblasts, astrocytes and other cells have a diameter of 60 Å (Ishikawa, Bischoff and Holtzer 1969, Bhisey and Freed 1971, Spooner, Yamada and Wessells 1971). The fibrils seen in the stereoscan image are probably aggregates of these fibrils. But other microfibrils are also known to be present in mammalian cells. The 100 Å diameter microfibrils are mainly located rather generally in the cell cytoplasm while microtubules of 270 Å diameter are seen to be centred round the region of the centriole and to radiate from it, at least in the case of macrophages (Bhisey and Freed 1971). These are generally increased during mitosis.

At this stage of our knowledge, it would be unwise to say which of these fibrillar systems is mainly responsible for the maintenance of cell shape between these groups, but it is clear that some type of microfibrillar organisation is most likely to play a part in maintaining the characteristic shapes of cells in culture.

Cell motility and cell shape

Living tissue cells are in a state of constant activity, particularly in the region of the cell surface complex. A complete understanding of the origin of cell shape must therefore depend not only on surface adhesions and a cytoskeleton but also on the interaction of the molecular structures responsible for these properties with the motive force for cell motility.

An examination of the subsurface structure seen in the region of an undulating membrane throws some light on this problem. In Pl. I 2 is shown a region of undulating membrane before etching; in Pl. II 9 is shown a similar region after etching. Evidence for the presence of ordered groups of microfibrils is seen in the long pseudopodial region in the lower part of Plate II. But in the peaks of the undulating region such regions are absent.

Microvillae, on the other hand, have often been studied in E. M. section. These long straight pseudopodia contain groups of microvillae lying parallel to the long axis. The irregular pseudopodia arising from the surface of the highly malignant glioma have spherical ends. This bulbous shape resembles closely the shape of a jet arising from a pure liquid, whose shape is determined by surface tension and by the momentum of the molecules of the liquid. (See for example the important topographical theory of Thom).

In this case, after ion etching, it can be clearly seen by comparing Plates I 5 and II 12 that there is a complete absence of detectable microfibular organisation in these pseudopodia. But it is clear that this loss of structure and orientation of cytoplasm is not accompanied by a decrease in the active movement of the cell surface complex, as can readily be seen in time lapse cine films of malignant cells. Light on this problem has been given by recent papers. Vasiliev, Gelfand, Domina, Ivanova, Kom and Olshevskaja (1970), studied the movements of fibroblasts in the grooves of fish scales. They show well organised contact guidance (Weiss 1962) along the grooves. After treatment with colchicine the cells show a more amoeboid type of locomotion and fail to maintain contact guidance. Freed, Bhisey and Lebowitz (1968) and Bhisey and Freed (1971) have described a loss of fan-like cell movement in macrophages and in HeLa cells, after treatment with colchicine or vinblastine. In the case of vinblastine the change is reversible; during treatment macrophages produce large irregular pseudopodia; cytoplasmic organelles stream into the pseudopodia, which never happens in untreated cells.

This change is accompanied by loss of organisation of microtubules around the centriole and the appearance of bundles of ~ 100 Å microfibrils in the plasma sol. These may be produced by the breakdown of microtubules; but the structure of the 60 Å microfibrils near the cell surface is preserved.

Evidence that these 60 Å microfibrils are similar to the actin filaments of muscle has been obtained by Ishikawa, Bischoff and Holtzer (1969) who have shown that they bind the heavy meromyosin head proteins from muscle to give rise to the

characteristic arrow-head pattern which can be produced when heavy meromyosin reacts with the I bands of striated muscle or with isolated actin filaments (Huxley 1963). Such microfibrils are found in the plasma gel region of fibroblasts, chondrogenic cells, nerve cells, and also in several types of epithelial cells. Gröschel-Stewart, Jones and Kemp (1970) have shown that an antibody to smooth muscle actinomyosin will, after fluorescent labelling, give rise to fluorescence in the region of the cell surface complex, in embryonic liver and other cells. This particular antibody inhibits Ca^{++} dependent ATPase activity of smooth muscle but does not affect the type of ATPase activity associated with ionic pumps in human erythrocyte ghosts which is Na^{+} K^{+} and Mg^{++} dependent. Combining this evidence with that from other work with plant cells (Jarosch 1956, Kamiya 1959) plasmodium (Wolhfarth-Bottermann 1961) a good case could be made for the presence in the plasma gel of many cells of 60 Å diameter microfibrils of actin-like protein and of ATPase activity similar to that of muscle actinomyosin. The fact that myosin has not been identified morphologically in E. M. sections does not exclude its presence in association with the actin-like fibrils because it is not possible to identify myosin morphologically in many types of smooth muscle, probably due to lack of a regular periodicity similar to striated muscle. Certainly the binding of heavy meromysin (HMM) in arrowhead form indicates that the subsurface microfibrils possess polarity. The two strands of globular actin in actin fibrils are known to be assembled with identical polarity.

As to the motive force for some local cytoplasmic and membrane movement, it was suggested by the author (Ambrose 1965) that this might arise from electroosmosis generated by electrical potential differences arising parallel to the inner surface of the plasma membrane. These potential differences were considered at that time, to be due to ionic pumps in nonuniformly spaced pores in the membrane which gave rise to local changes of potential along the membrane surface. In view of the known lability of membrane structure this arrangement of pores at the surface provided a rather unsatisfactory model. A much improved model has been proposed by Hejnowicz (1970) based on electroosmotic forces generated on microfibrils embedded at the inner surface of the plasma gel. Conformational changes propagated like waves along the fibrils have an electric component. This gives rise to net electroosmotic flow in one direction in the boundary layer of fluid when the waves are propagated in series. The potential change is derived by pumping cations into and out of the fibrils. The magnitudes of the energy changes and electroosmotic rates of flow are found to be of the correct magnitude to fit the known biological phemonena in cyclosis and other types of protoplasmic movement.

In Fig. 4 is shown an alternative but basically similar model for cytoplasmic movements generated at the boundary between plasma gel and sol based on the various known molecular events in muscular contraction.

(1) The plasma membrane now has the function of initiating the entry of Ca^{++} ions into the plasma gel in a general sense (not localised as in the previous model

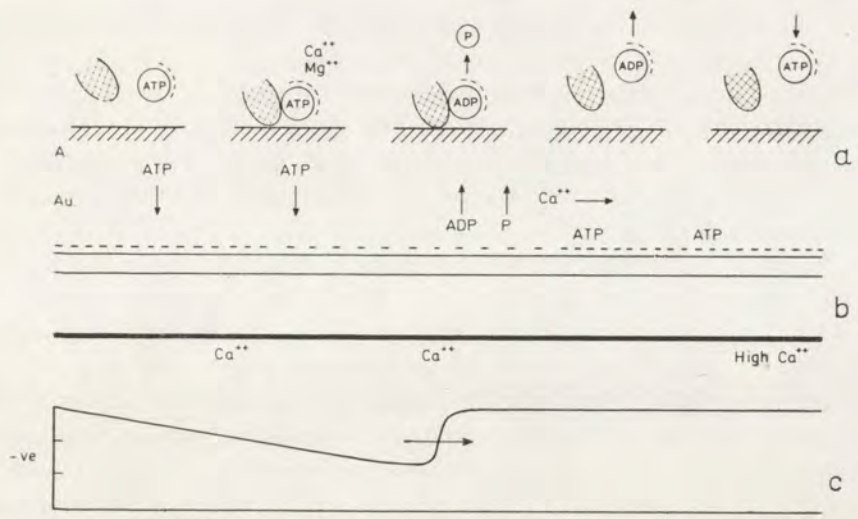

Fig. 4. a — stages in the hydrolysis of ATP to ADP and inorganic phosphate in the ion action filaments in the presence of heavy meromysin, b — model for the generation of a progressive electrical disturbance along an actin-like microfibril. High Ca^{++} — Calcium concentration in the external medium. ATP — region of adsorbed ATP. Ca^{++} — Calcium ions being carried by electroosmosis towards regions of unhydrolysed ATP. $\uparrow P$ — P^- ions being lost from the surface. $\uparrow ADP$ — ADP ions being lost from the surface, $ATP\downarrow$ — ATP ions being slowly readsorbed, c — form of the wave of changing Zeta potential generated by the progressive hydrolysis of ATP

of Ambrose 1965). This varies the Ca^{++} concentration between 10^{-7}M (switched off) and 10^{-5}M (switched on) as in the sarcolemma of muscle.

(2) A wave of hydrolysis of ATP (Ambrose and Easty 1970) occurs along the fibril involving association of heavy meromysin with the actin-like filament. This leads to changes in the Zeta potential at the surface of the fibrils due to net changes in the number of negatively charged groups adsorbed at the surface of the fibril. This is due to rapid diffusion of myosin phosphate and later of ADP from the surface. ATP then readsorbed more slowly onto the fibril which is then restored to its original Zeta potential and is ready for triggering for the generation of a fresh wave.

(3) This type of disturbance can be self-propagating since the potential gradient itself will draw divalent Ca^{++} ions more rapidly then monovalent K^+ or Na^+ towards the region of unhydrolysed ATP. In this way the Ca^{++} ion concentration can increase progressively along the fibril to the level required to trigger hydrolysis of ATP. The potential gradient in the region of hydrolysis will draw Na^+ and K^+ ions by electroosmosis carrying the associated water in bulk flow.

The system can be likened to the pendulum of a clock in which the general movements of the liquid is maintained by the successive application of waves of electrical potential travelling along the fibril, at approximately the same velocity.

Mechanical models, based on wave-like movements of the fibrils (Jarosch 1964) and on changes of angle of attachment of heavy meromysin to actin (Pringle 1968), have been suggested. Electrical forces operating on charged groups will operate over a much greater bulk of the liquid and will be far more effective in producing bulk flow, as is observed in protoplasmic streaming, than could be achieved by the short range forces operating over a few Ångströms that would be effective in the use of a mechanical model. This is not to say that mechanical displacement of the fibrils may not be observed in extreme cases of protoplasmic movement, as in the rotating fibrils observed by Jarosch and Kamiya in *Nitella*. But, according to the model described by Hejnowicz and the model shown in Fig. 4, the motive force for cytoplasmic movements, including wave-like movements has an electrical origin. Reversible expansion and contraction of cell membranes has already been shown to take place in the presence of combinations of polvalent anions and cations. Contraction occurs with ATP and Mg^{++} ions (Korohoda, Ambrose and Forrester 1967). Local transient shape changes on a microscale as seen in Plate I 2 may well affect the permeability of the plasma membrane to Ca^{++} ions and hence lead to ATP hydrolysis.

General conclusions

The suggestion was made (Ambrose 1961), from studies with the surface contact microscope, that membrane undulations similar to those seen in Plate I 2 also form on the lower surface of the cell in contact with the solid substrate.

This can in fact be seen in the stereoscan picture of the contacts between a fibroblast and a glass surface in Plate I 10. Studies by Korohoda (1971) of the movements of fibroblasts along fine glass filaments have made it possible to visualise these movements on the lower surfaces of cells directly. This basic mechanism of locomotion of fibroblasts as a consequence of intermittent contact with the substrate generated by membrane undulations is now well supported by these various types of observation.

The form of the membrane movements involved appear to depend on the cell type. A detailed analysis of the generation of the single crests or lamellipodia seen on the leading edge of fibroblasts has been made by Abercrombie, Heaysman and Pegram (1970 a, b). This work shows that there is a considerable local movement at the cell surface, particles being carried backwards for a limited distance during the formation and readsorption of the lamellipodia. They suggest that new membrane may be produced during the initiation of lamellipodia which is readsorbed a short distance further back. The cell contacts to the glass which form on the lower surface are probably stronger in this region and have a longer duration than those seen in other parts of the underside of the cell with the surface contact microscope. These membrane movements, which initiate cell spreading, probably provide the first stages in establishment of cellular morphology in culture. In those cells which

contain many microfibrils, the flow process associated with cell spreading leads to parallel orientation of the microfibrils which are then influential in maintaining a permanent extension of the cell. In those cells which are less adhesive to glass but which contain many microfibrils, these may associate in bundles, so leading to the star-shaped form seen in astrocytes. Microtubules may also be oriented below the outer layers deeper in the cytoplasm (Spooner, Yamada and Wessells (1971).

These are comparatively simple concepts; they depend on relative changes in the proportions of cellular products to give rise to cellular characteristics i.e. the proportion of the various liquid components of the cell membrane, the proportions of the surface coat materials which determine adhesion, the proportions of the various types of microfibrils and their state of aggregation.

Nevertheless, such simple properties must be extremely accurately controlled in a time sequence of cell cycles and cell generations during growth and morphogenesis in living organisms. We are just beginning to study the way in which these cell surface properties, governing cell shape, influence the association of cells with other tissues by three-dimensional time lapse filming of organ cultures. The development of organisms may perhaps be compared to a musical composition. The number of notes in the octave is limited but their combinations and sequences in time are almost unlimited. So far, the search for highly specific biochemical studies responsible for the initiation of stages in development have proved elusive. It is distinctly possible that an understanding of these processes, both in normal and in malignant growth, will in the future depend on a better understanding of the supermolecular organisation of cells of the type considered in this paper.

Acknowledgements

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Summary

Studies by Stereoscan Microscopy and time lapse ciné filming of normal and malignant fibroblasts, and of non-malignant and malignant brain astrocytes indicate that the presence of fan-like membranes and elongated pseudopodia is associated with the orientation of sub-surface microfibrils. The shape of isolated cells in monolayer culture depends on adhesiveness to the substrate on sub-surface microfibrils, on microtubules and on cell membrane motility.

The role of these various factors in determining the characteristic shape of various cell types is discussed.

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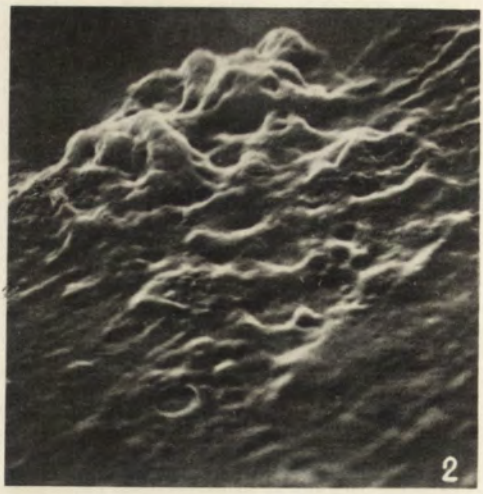
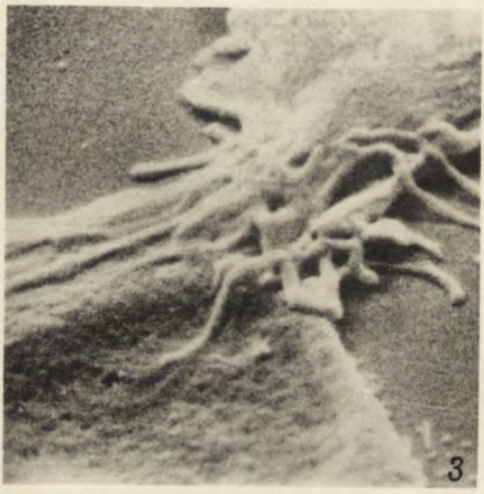
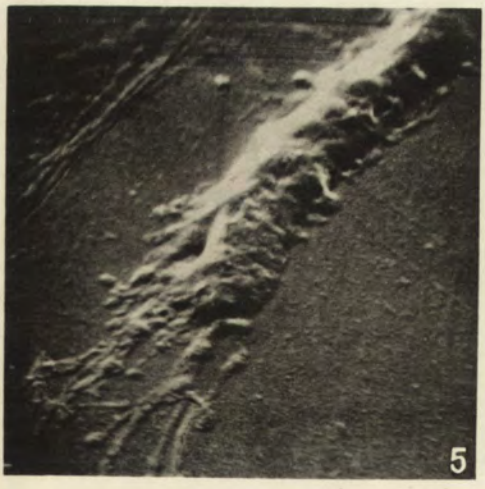
EXPLANATION OF PLATES I–II

1: Lymphocyte showing millipodia in contact with glass. Erythrocyte in background.
2: An undulating membrane on the upper surface of a fan-like membrane of an astrocyte
3: Microvillae in a region of two cells beginning to form contacts
4: Microvillae on the lower surface of a fibroblast in contact with glass
5: Bulbous type of pseudopodia produced by a malignant glioma cell
6: Extremely elongated star-shaped pseudopodia in non-malignant astrocytoma cells
7: Fibroblast surface etched deep into the plasma gel to reveal the reticular appearance of the plasma sol below
In the central band, etching is not so deep and bundles of microfibrils can be seen
8: As above, but etched to reveal a region of well orientated microfibrils embedded in the plasma gel
9: Ion etched region of the fan-like membrane of an astrocyte. Bottom. End of long pseudopodium show microfibrils lying along the long axis
Top. Region of membrane undulations similar to Plate I 2. No general fibrillar organisation detected in the crests of the waves
10: Cell body of glial cell after ion etching showing aggregate of fibrils 5000 Å in diameters
11: Rear region of retraction of a glial cell showing attached millipodia
12: Region of bulbous pseudopodia similiar to Plate I 11, but after ion etching showing ancorphores appearance of subsurface material







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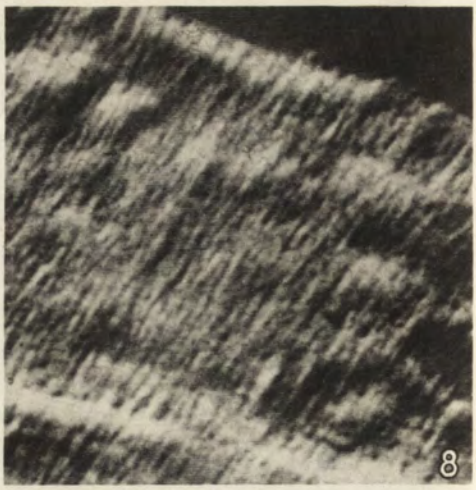

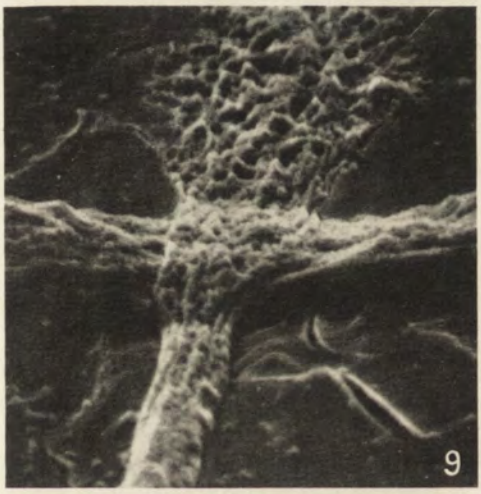

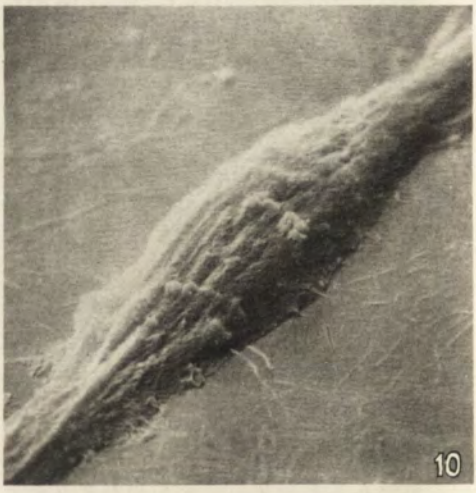

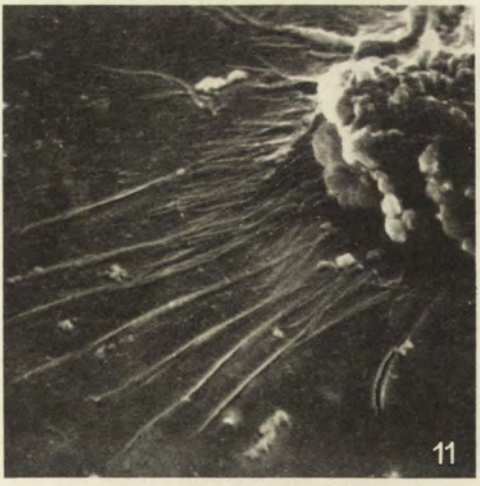

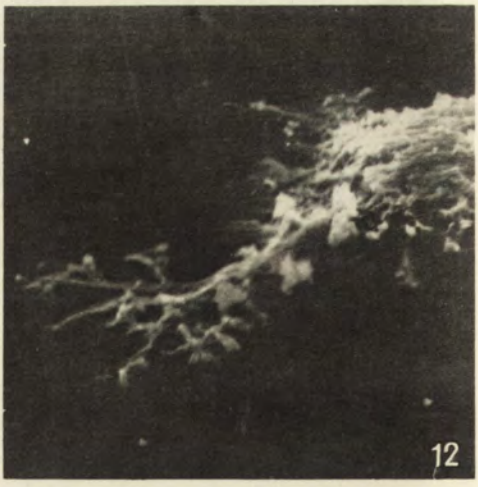

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The participation of rotating fibrils in biological movements

The object I would like to discuss here is the mechanism of biological movements. But I shall not deal with the molecular basis of this motion, that is the problem of transformation of chemical to mechanical energy and the conformational changes in the protein molecules connected with this process – I wish to discuss the question how biological movements are generated at the microscopic and submicroscopic level.

The recent conceptions on cellular functions are widely influenced by the results of electron microscopy and biochemistry. But we should not overlook that these methods deal with non-functional material and that the strong mechanical tensions produced inside of the motion-active molecules do them not allow to become prepared and fixed in their native structure. For example there is some evidence (see below) that what appear as globular subunits in electron micrographs of bacterial flagella cannot show this configuration in the native state. Conclusions should be drawn first of all from living material. Mainly on the basis of light-microscopic observations of high resolution I supposed rotating protein helices as the motor-organelles of some biological movements (Jarosch since 1963), but this concept has been widely ignored. Therefore I shall discuss here some observations more in detail.

Bacterial flagella

These organelles normally show helical size. We can observe very distinctly on resting cells in the dark-field, that the coils move as helical waves from the base to the tip of the flagellum (small arrows in Fig. 1). The waves resemble to the apparent movement of the coils of a rotating helix. But Bütschli (1883) has suggested another interpretation for the helical waves at the flagellum of the eucaryotic cell. He assumes a contracting region travelling in a spiral course around the flagellum. This idea has been applied to the bacterial flagellum by Reichert (1909) and recently discussed by Lowy and Spencer (1968) in connection with the arrangement of the globular subunits demonstrated with the electron microscope in negatively stained preparations. Detailed observations with darkfield- and phase-contrast-microscopy on li-

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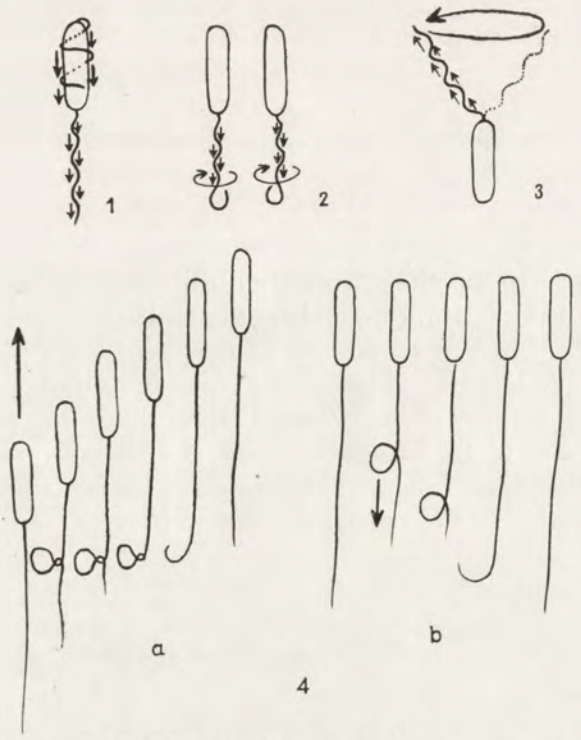

Fig. 1. Typical behaviour of the bacterial flagella. One flagellum is wound about the cell. The coils of the helical flagella move as indicated by the arrows
Fig. 2. The tip of the flagellum shows a loop similar to the hook of a coat-hanger. Its rotation demonstrates the true rotation of the flagellar fibrils
Fig. 3. The helical coils move also (small arrows) during the funnel-shaped motion of a bent flagellum
Fig. 4. a — a non-helical flagellum forms a loop. The cell slides foreward and the flagellar fibrils (in contact with the glass-surface) move through this loop, b — when the cell does not move the loop moves to the tip of the flagellum

ving bacteria have shown that this model is wrong and that the bacterial flagella are actually rotating helical fibrils (Doetsch 1966a, 1966b, Doetsch and Hageage 1968, Jarosch 1963, 1967, 1969). These observations are as follows.

(1) The rotation is very well demonstrated when the flagellum shows a loop at its tip. This loop is similar to the hook of a coat-hanger and appears frequently at different cells (Fig. 2). An explanation of this rotation by the model of contraction would appear to the author similar to an explanation of the revolution of a car-wheel by contractions of the car axes.

(2) An apparent rotation of the flagellum caused by an active bending motion in the basis of the flagellum (see Fig. 6 of Lowy and Spencer 1968) would not cause a movement of the helical coils in the terminal derection. The shifting of coils (small arrows in Fig. 3) is very distinctly on resting cells showing this type of flagellar movement.

(3) Certain bacterial cells slide with a non-helical flagellum at the cover glass. These flagella sometimes form a loop like a telephone-cable. The cell slides foreward and the flagellar substance moves through the loop (Fig. 4a) or – when the cell remains at the same place – the loop moves to the tip and disappears there (Fig. 4b), although the flagellum does not show any bending motion! It is impossible to interpretate this phenomenon by contractions. The behaviour is typical for a flexible rotating coil (see Fig. 21 of Jarosch 1965).

(4) The observation just described and the sliding of the cells over the glass-surface without bending and waves (Fig. 4 and Fig. 5) demonstrate an active shearing force at the surface of the flagellum. The movement of adhering ink-particles along the flagellum described already by Pijper (1957) and perhaps the transport of phage-

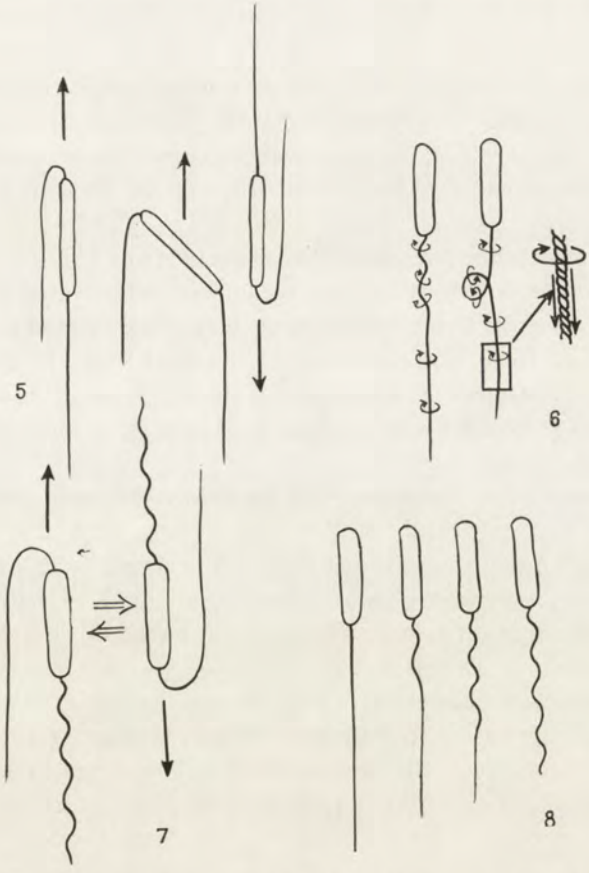

Fig. 5. Sliding of bacterial cells (arrows) within the aid of non-helical flagella which are in close contact with the glass-surface. They generate an active shearing force
Fig. 6. The active shearing force interpreted as the result of the rotating flagellar fibrils. Because of the quick rotations the submicroscopic coils of the flagellar fibrils generate a shifting effect
Fig. 7. Transformation of the flagella from helical to non-helical and vice versa
Fig. 8. The transformation progresses from the base to the tip of the flagellum

particles (Schade et al. 1967) indicate also the shearing force. We may explain this force by the apparent movement of the submicroscopic helical coils of the rotating flagellum (Fig. 6). It is clear that such a shifting-effect may be generated only by a rather distinct helical structure, which has been described on many cases for the submicroscopic structure of the flagellar surface or sheath (Starr and Williams 1952, Labaw and Mosley 1954, 1955, Swanbeck and Forslind 1964, Lowy 1965, Lowy and Hanson 1965, Sleyter et al. 1967). A longitudinally "lined" or hexagonally "beaded" arrangement of globular subunits on the surface of the flagellum (see the models of Kerridge et al. 1962, Lowy and McDonough 1964, Lowy and Spencer 1968, Tauschel and Drews 1970) would hardly cause a shifting-effect.

(5) Bent flagella frequently show a jerky- or pulsative motion. This is typical, e. g. for an elastic rotating wire that forms a bend or a loop. The internal torsional resistance is here overcome in pulsative revolutions (film on the flagella motion of *Spirillium volutans*).

From the described observations there is not the smallest doubt that bacterial flagella are truly rotating helical fibrils. According to the stroboscopic measurements of Metzner (1920) the speed of the revolutions may show values up to 90/sec. Similar and higher values have been calculated, also for the revolution of th DNA-double-helices during their reduplication (Cole 1962,) and for the rotating fibrils of the active protoplasmic streaming (Jarosch 1964b).

Another question is: where in the flagellum is the energy for the revolutions generated? Following the traditional concept the author believed at first (1963) that the active torsional force is generated along the total length of the flagellum. But after detailed observations and experiments with models he inclines now to the concept of Doetsch (1966a, b) who assumed the active force for the rotation only in the basis of the flagellum.

It is important in this connection that the passive torsional force [1] which arises in the flagellum by the basically produced revolutions may effect a change in the flagellar size (pitch and diameter) and rigidity. An extreme change takes place when a helical flagellum is transformed into a straight one (Fig. 7, Pl. I 1, 2). This transformation progresses from the basis of the flagellum to the tip (Fig. 8). It seems from these observations that the helical size of the flagellum is due to an inside tension and that this tension is changed by a passive torsional force. A glycerol-treatment (40% and higher) changes also (but irreversibly) the flagellar size from helical to straight or to a denser coiled helix (compare Pl. I 3). This demonstrates that a glycerol-treated protein does not absolutely retain its native structure (release from tension?).

The flagellar bundle

The flagellum we may see in darkfield- and phase-contrast-microscopy is actually a bunch of a few or many single flagella. The isolation of the single flagella in dried

[1] We must distinguish between an active torsional force, which may be produced by a

preparations is an artefact and takes normally not place in the living state (Neumann 1925). But a splitting is sometimes found in the living state too (Pijper 1957) especially in very thin preparations, e. g. of *Spirillum volutans* (Pl. I 4). The single flagella assemble and move together because their apparent waves come in phase. The single fibrils rotates in such a waving helical bundle as indicated by the arrows R_1 in Fig. 9. The friction-forces produced here by the twisting-movement of the single fibrils presumably facilitate in flattened flagella the splitting of the bundle.

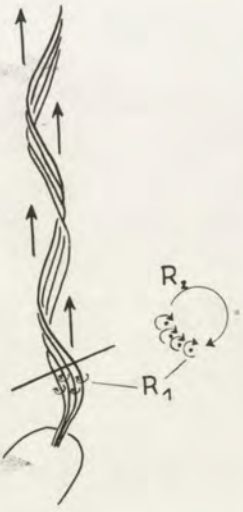

Fig. 9. Schematic drawing of a bacterial flagella bundle. The revolutions of the single helical flagella are indicated by the arrows $R_1 \cdot R_2$ indicates the rotation of the bundle

Gliding of bacteria and spirochaeta

A good indicator for an active or passive torsional force in a thread is its twisting about itself, (Pl. II 5–13). When we see such a behaviour we may be sure that there are rotating fibrils involved because a contraction may never generate this configuration. Gliding bacteria, blue-green algae and spirochaeta frequently show this configurations (Pl. II 5–7). Their characteristic bending-movements, which we can imitate rather exactly by model-experiments (Jarosch 1967, film), also indicate the torsional force. The presumable situations of the rotating fibrils for the main types of movement are shown schematically in Fig. 10.

The eucaryotic flagellum

The flagellum of eucaryotic cells is a bundle of fibrils showing the well known 9+2 pattern. We observe helical or uniplanar waves. But these bendings appear not so rigid as at bacterial flagella. Size and behaviour varïates widely.

An important property of protein-fibrils showing superposed coils, is their abi-

conformational change in the protein-molecule and causes a rotation, and the passive torsional force, which arises in a rotating fibril by the resistance-force of its environment.

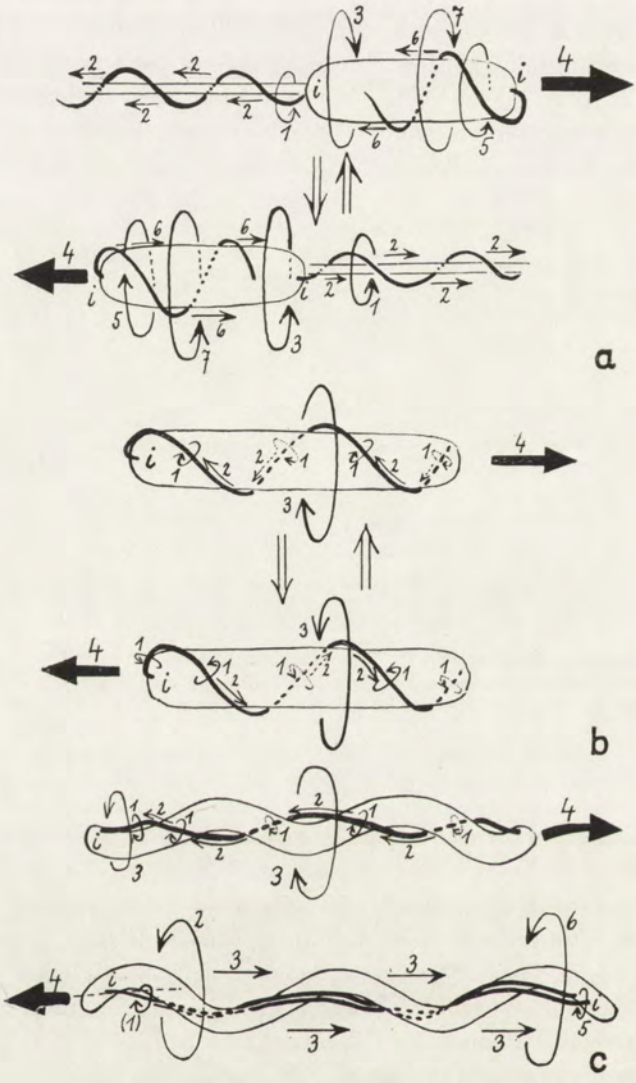

Fig. 10. Situation and behavior of the rotating fibrils for the main types of procaryotic cell-movements, a — swimming bacterial cell, arrows 1 and 5 — rotation of the flagella, arrows 3 and 7 — resulting rotation of the cell, arrows 2 and 6 — apparent waves of the flagella, arrow 4 — resulting movement of the cell, b — sliding cells (blue-green algae, bacterial cells and *Spirochaetae*) arrows 1 — rotation of fibrils (rapidosomes), arrow 3 — resulting rotation of the cell, arrow 2 — shifting-direction of the submicroscopic coils at the fibrils (can move slime), arrow 4 — resulting movement of the cell, c — swimming *Spirochaetae*, arrows 1 and 5 — rotation of fibrils, arrows 2 and 6 — resulting rotation of the cell, arrow 3 — apparent waves of the cell-coils, arrow 4 — resulting movement of the cell, i — possible site of fibrillar insertion. For more details see Jarosch (1967)

lity to rotate without apparent waves of these coils. The big helical coils are flexible and do not deform although the fibrils rotate. This property is exactly imitated by a model of a supercoil which consists of two or more intertwined helices showing a small pitch-difference (Jarosch 1963, 1964a). We can imitate many properties of the eucaryotic flagellar movement when such a model rotates in honey (Jarosch 1968 b), where the frictional resistance is high (Fig. 11). The flagellar waves are here imitated by the friction-retarded movement of the supercoils (arrows S in Fig. 11d). The frictional resistance is at all a very important factor for the rotating fibrils (see

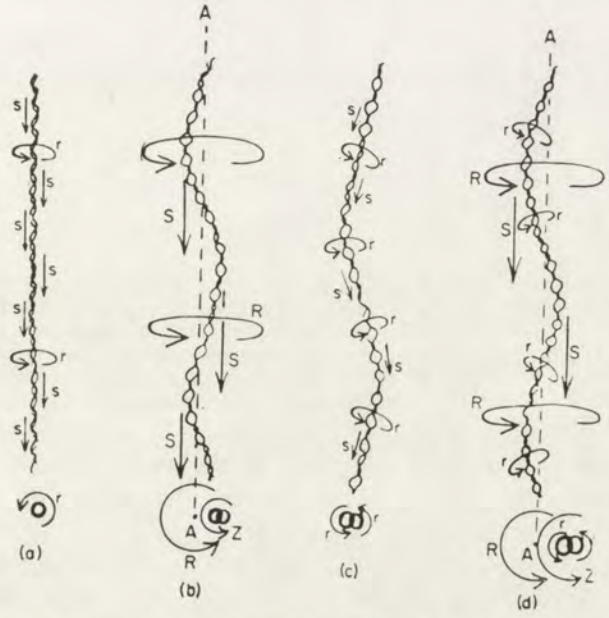

Fig. 11. The different possibilities for the motion of supercoils when rotating in a viscous medium, a — no tension, straight fibril (no pitch-difference between the two intertwined helices), rotation (arrows r) with apparent movement of the small coils (arrows s). The fibril vibrates only, b — strong tension, helical fibril (pitch-difference), rotation (arrows R) with apparent movement of the supercoils (arrows S). The rigid supercoiled fibril rotates. A — axis of the helix, Z — rotation of the two connected helices, c — week tension, helical fibril (small pitch-difference or less resistance of the medium), rotation (arrows r) with apparent movement of the small coils (arrows s), but without movement of the supercoils (inner bending), d — the motions as described in b and c take place at the same supercoil. The rotation of the fibril (arrows r) is quicker than the rotation (arrows R) and apparent movement appear of the supercoils (arrows S). They are retarded by the frictional resistance of the medium. (According to Jarosch 1968 b)

below). When a beating flagellum is flattened the travelling waves become very slowly and jerky because the frictional resistance strongly arises by the compression. Under these conditions each bend that arises at the flagellum is apparently a factor of retardation. It disappears when it arrives at the tip of the flagellum. This is accompanied by a jerky release from the tension of the whole flagellum, because other bends of the flagellum may take part on this jerky motion (film on the flagellar motion of *Tetramitus*

sp. and *Synura bioreti*). The longer one of the two flagella of *S. bioreti* beats in an uniplanar way. The tip of the flagellum becomes a helix when the cell is demaged by flattening (Fig. 12 a). Both, the uniplanar waves of the base and the helical waves of the tip progress synchronously in jerkies (arrows in Fig. 12). This demonstrates fibrils which rotate in the total length of the flagellum. In one observed case a slender stiff helix projected out of the tip of the uniplanar flagellum (Fig. 12 b). It rotates (!) synchronously with the jerky motion of the uniplanar waves.

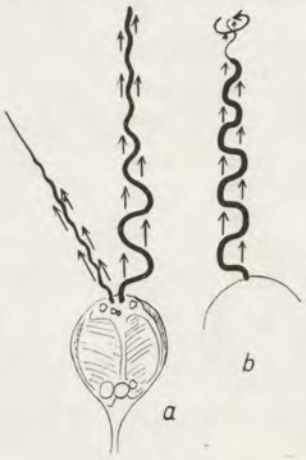

Fig. 12. Compressed *Synura bioreti*, a — the smaller flagellum shows helical the larger uniplanar waves. The tip of the larger flagellum has become helical but all coils and bendings of the flagellum move synchronously in jerkies. b — a slender helical coil projects out of the uniplanar flagellum. It rotates synchronously with the movement of the uniplanar waves

The jerky waves may be also imitated by the model rotating in honey (film). Sometimes we may observe a damaged flagellum that is bent off and twists about itself (film on the flagellar motion of *Carteria*).

We may suppose the eucaryotic flagellum as a bundle of rotating fibrils showing the possibility for generating superhelices at the single fibrils. Helical waves are supposed as the retarded apparent waves of the rotating superhelices (arrows S in Fig. 11 d). The uniplanar waves may arise by at least two superhelices rotating in opposite directions and therefore showing opposite coiling sense — as the model shows too [2]. The intimate mechanic combination of two in opposite directions coiled fibrils — which we must assume in the flagellum — generates uniplanar bendings (for details see Jarosch 1970). The uniplanar waves thus depend on the simultaneous revolutions of the supercoiled fibrils in both directions.

Rhizopodial movements

There are several observations which indicate rotating fibrils in the rhizopodial cell. I am brief here because I have described some of them in recent papers (Jarosch 1968 c, 1971 a).

[2] Because of the friction, which effects a passive torsional force the superhelix coils always in the opposite direction of the rotation. It coils counterclockwise when it rotates clockwise. It coils clockwise when it rotates counterclockwise.

(1) Slender processes at the rear of small amoeba rotate. The processes may be generated by an adhearing of rear-substance on the glass-surface. They are drawn out by the progressing-motion of the creeping cell. After tearing away they become bent and rotate (about 2 revolutions/sec). Then they are melted again in the rear (Jarosch 1971 b).

(2) The tail or the whole cell of small amoeba may show rotational movements.

(3) Filopodia may twist about itself and branch (Pl. II 12) indicating torsional forces.

(4) Slender filopodia vibrate during their out-growing.

(5) Filopodia, axopodia and small lobopodia produce active shearing-forces. The cells move when these organelles are in contact with the glass-surface (compare the gliding of bacterial cells with the non-helical flagellum).

(6) Certain small amoebae show helical pseudopodia with waving-motion (Bovee 1964). These pseudopodia apparently contain bundles of helical fibrils which rotate similar to the flagella in a helical flagellar bundle of a bacterium (see above).

Active protoplasmic streaming

We have described the active bacterial flagellum as a bundle of rotating helical fibrils. It produces a streaming in the surrounding medium, e.g. indicated by ink-particles, when it rotates quickly. An ink-particle that comes in close contact with a quickly rotating cell of *Spirochaeta biflexa* is shifted too (Fig. 13 b). *S. biflexa* rotates quickly on the same place when suspended in water. Its helical body is very small (pitch about 0.3 μ). Thus the quickly rotating coil appears as a bright line in the dark-field. This observation resembles to a similar shifting-effect that appears along protoplasmic fibrils (Fig. 13 c). The resolution-power of the light-microscope is to small in order to observe the helical structure of these fibrils. Two kinds of fibrils

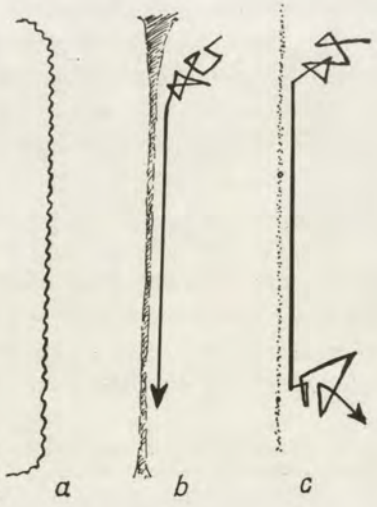

Fig. 13. *Spirochaeta biflexa*, a — resting, b — in quick rotation, shifting an ink-particle, c — an endoplasmatic particle is shifted by a fibril of the characean protoplasm

have been described in electron microscopic preparations. Both, the thicker "tubules" and the thinner actin-like fibrils have a helical structure and both are described as being involved in the generation of movements (e.g., Roth 1964, Porter 1966). The rate of the endoplasmatic movements variates widely, and this may depend on the sort of involved fibrils. The tubular fibrils (MT of the mitotic apparatus, neurotubules, MT in the axopodia of *Heliozoa*, etc.) seem to move the particles slower than the actin-like fibrils[3].

The extremely quick protoplasmic streaming in the characean cell is obviously caused by actin-like fibrils joined together in bundles of about 50–100 as was shown by Nagai and Rebhun (1966). These fibrils already described with the light-microscope (Jarosch 1955, 1956, 1958, 1964, Kamiya 1959, 1962, Kuroda 1964, Kamitsubo 1966) show similar properties we know from the bacterial flagellum: movement by active shearing forces and transversal waves. We may conclude that they are bundles of quickly rotating fibrils. There arise many questions in connection with this concept and I am not able to discuss all this problems here, but I would like to contribute something to the problem of friction.

The frictional resistance is an important factor because it limits the revolution-speed of a fibril. The relation between the rate of protoplasmic streaming and temperature (see Kamiya 1959) is apparently a simple physical relation between viscosity (frictional resistance) and temperature (see Jarosch 1964 a). The friction also limits the length of a fibril rotating in a viscous medium. The lipids inside of the protoplasm probably work as a lubricant (Jarosch 1966). That can be demonstrated by the flattened flagella of compressed *Tetramitus*-cells. They are hindered to beat and move in jerkies. But sometimes a flagellum gets into the protoplasma and then it beats there fully continuously. Inside of the protoplasm there is apparently less friction.

Another problem is the relation between frictional resistance and the structure of the rotating fibril. The smaller the length, the diameter and the diameter of the primary coils (diameter of wire) of a rotating helix is the quicker may be its speed of revolution (unpublished results of model-experiments). Model-experiments with wire-helices have also shown that tubules or densely coiled helices produce much more friction, when they rotate in honey than stretched helices (Fig. 14). This may be one reason for the slower motion of microtubular fibrils in comparison with the actin-like fibrils.

Finally I want to discuss the possible "summarizing-effect" that results during the cooperation of two or more rotating fibrils. The speed of a particle which is shifted by the apparent waves of a single rotating helix is firstly limited by the frictional resistance of the medium (dependence on the particle-size, smaller particles move quicker than larger ones). Secondly the speed depends on the contact of the particle

[3] Actin-like fibrils may also exert a hydrodynamic pressure on the endoplasm which brings forth the passive streaming in *Amoebae* and myxomycete-plasmodia (see Kamiya 1968). This kind of movement is here not discussed.

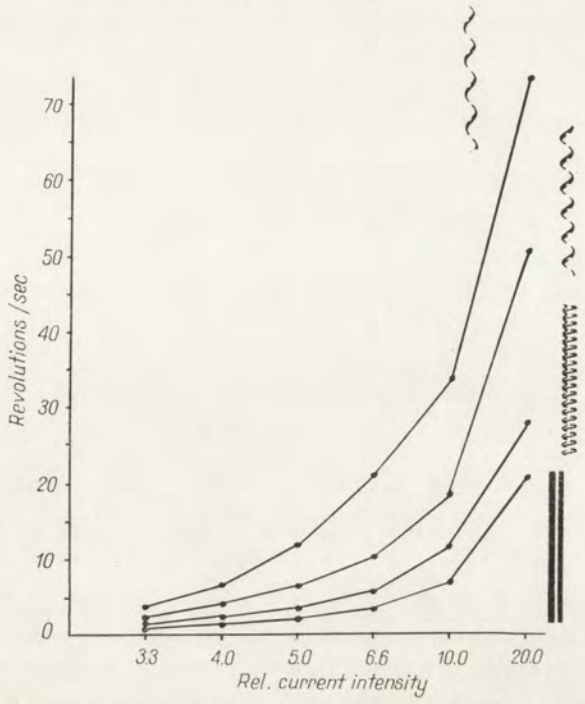

Fig. 14. Relation between the revolution-speed of helices rotating in honey and their coiling-density. The tubular helix below shows the greatest frictional resistance

with the rotating fibril. A better contact must increase the speed. When the particle gets contact with two or more fibrils working in the same direction, its speed will therefore increase too. This may be demonstrated under simplified conditions by an apparatus shown in Fig. 15. Long screw are brought in rotation by dropping weights.

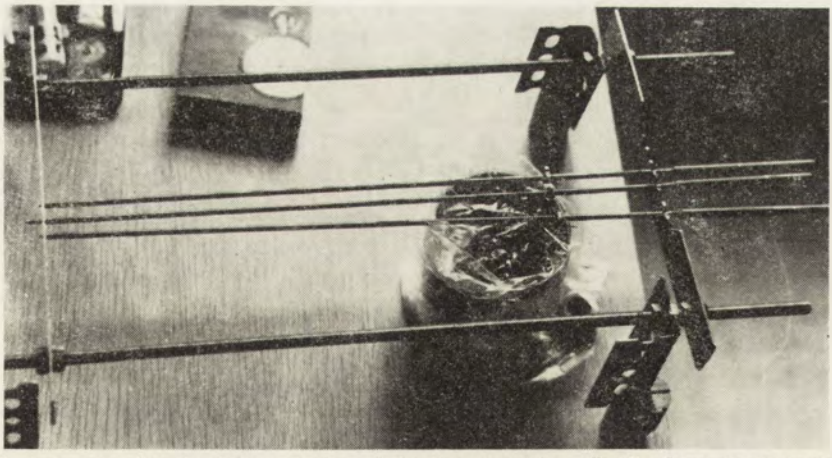

Fig. 15. Apparatus for the demonstration of the "summarizing-effect" of two or more rotating screws. For details see the text

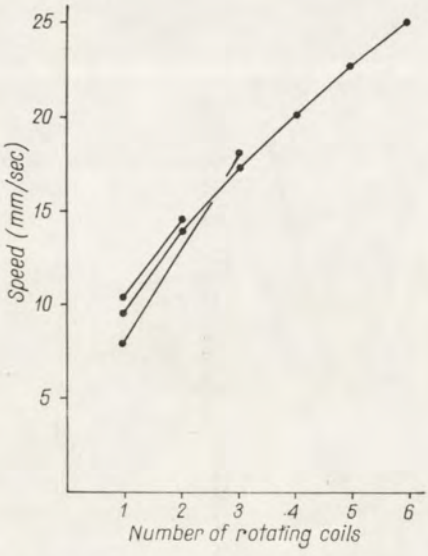

Fig. 16. Dependence of the speed of a shifted rubber-ball on the number of rotating screws

The screws shift nuts which may be mechanically connected by needles with a rubber-ball. The rubber-ball moves in a vessel filled with honey. The speed of this movement has been measured. It depends on the number of rotating screws which are in connection with the rubber-ball (Fig. 16). This would mean for the active protoplasmic streaming, the more rotating fibrils shifting in the same direction are present, the quicker is the speed of the movement.

Conclusion

I have tried to demonstrate here the occurrence of rotating fibrils in different cells and their involvement in the generation of movements. Thus we should accept the cellular dynamics not only at the level of smaller molecules and globular subunits, we should consider the living protoplasm as a system of fibrilar macromolecules under high tension which are operating by means of their revolutions at the cellular level too.

Summary

The flagellar fibrils of bacteria do not contract but rotate. The transversal waves, their pulsative character at bent flagella, the active shearing forces and the winding about itself are characteristic mechanic features of rotating helical fibrils. According to the theory of fibrilar contraction intricate additional assumptions are necessary to interprete these phenomena. Similar processes appear also at eucaryotic flagella, various pseudopodia and endoplasmatic

fibrils. Therefore it is suggested that their microtubular or actin-like fibrils rotate. The speed of a rotating fibril is primary limited by the frictional resistance. But it may increase when several fibrils are combined.

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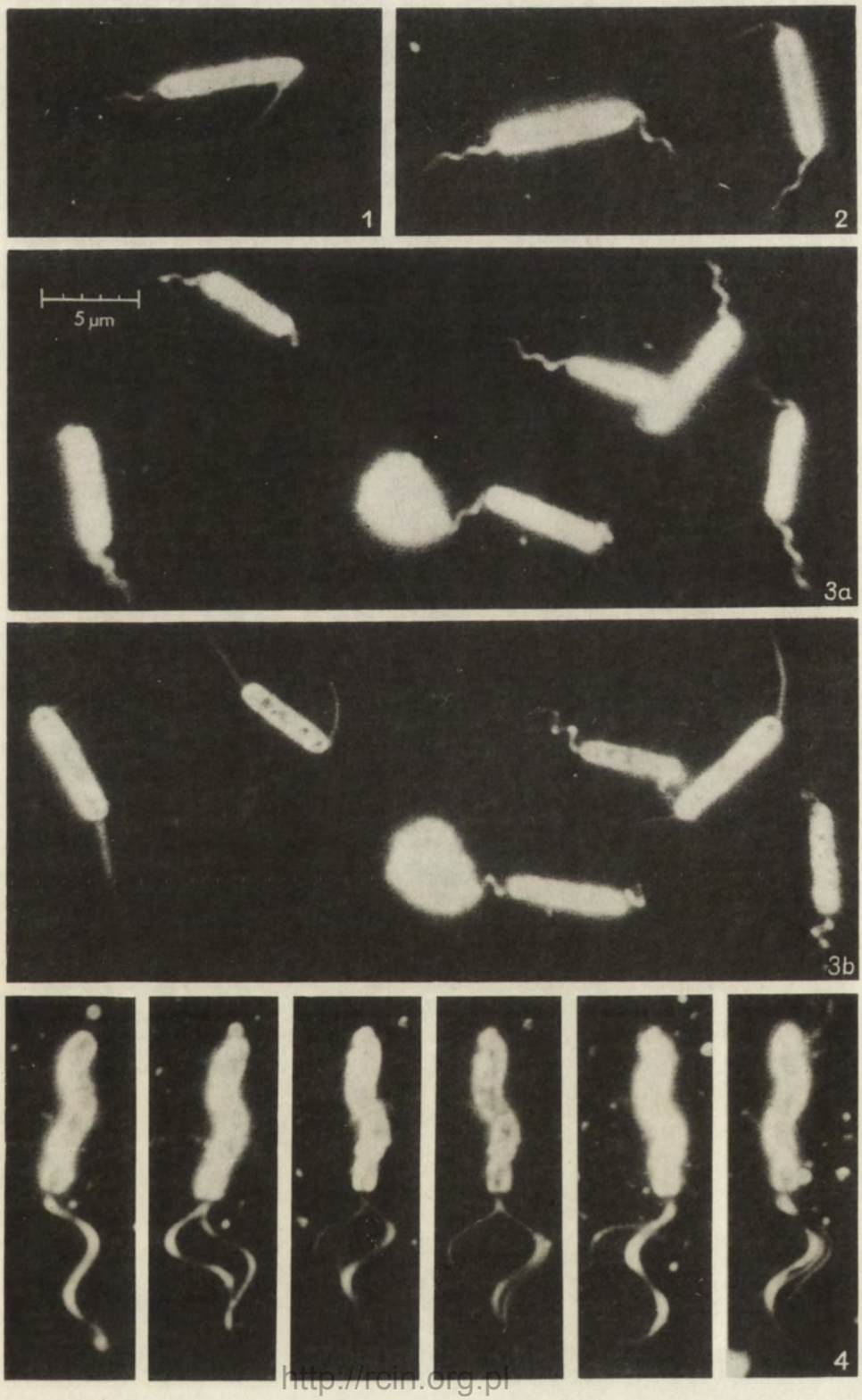
EXPLANATION OF PLATES I–II

1, 2: Living bacterial cells found in the mud of freshwater showing the described transformation of flagella (dark-field)
3: The same cells living (3 a), and dead, after treatment with 40% glycerol (3 b). The flagella have irreversibly transformed from helical to straight or to a coil with a larger diameter
4: A compressed cell of *Spirillum volutans* showing different splitting-states of the flagellar bundle
5–13: Different examples for the "twisting about itself" indicating fibrils which generate torsional forces. 5—*Spirulina* sp., 6—*Oscillatoria* sp., 7a—*Spirochaeta* sp., 7 b—Imitation by a wire-coil, 8—A plasmodial thread (from Kamiya and Seifriż 1954), 9—The "wicks" of microvilli from human intestinal cells (electron micrograph from Overton et al. 1965), 10—A chromosome of a human leucocyte (electron micrograph from Yasuzumi et al. 1951), 11—λ DNS prepared at low ionic strength (from Bode and MacHattie 1968), 12—A slender filopodium of a marine rhizopodial cell, 13—Characteristic branching of "silver lines" in the surface of *Colpidium* sp. (from Foissner 1969)



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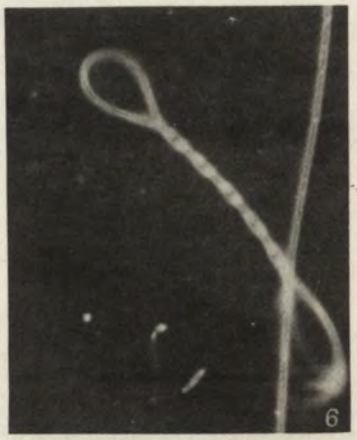

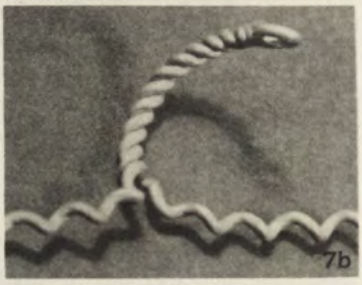

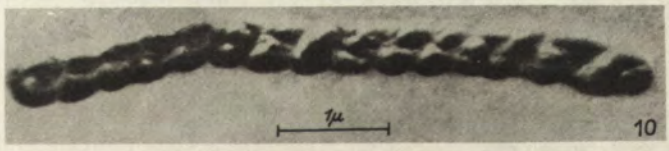

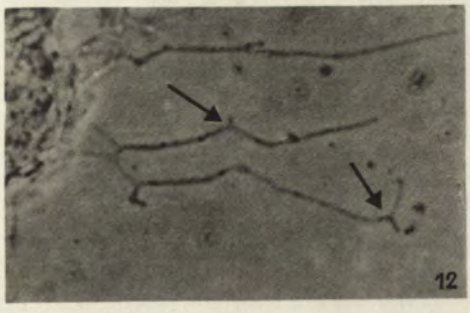

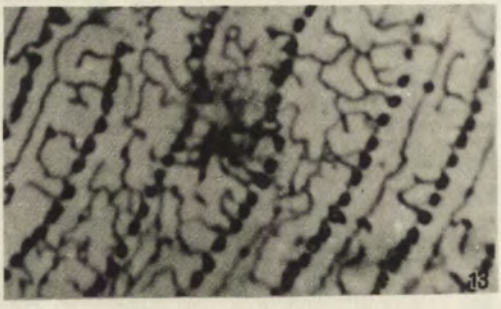

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Comparative studies on actomyosin-thread models of muscles and of myxomycete plasmodia. Their significance in the contractile mechanism of primitive motile systems

The acellular slime molds are one of the most favoured objects for the study of amoeboid movement and protoplasmic streaming. The species *Physarum polycephalum* is especially easy to handle in different experiments elucidating the principles of motive-force-generation and topographical distribution of contractile structures in primitive motile systems. In addition, this slime mold is characterized by high velocity of protoplasmic streaming and transport of a large mass of protoplasm. For biochemical investigations it is possible to culture large amounts of *Physarum polycephalum*, obtaining more than one pound within a few days.

The plasmodia of *Physarum polycephalum* show a net-like growth pattern (Pl. I 1) consisting of a multinuclear protoplasmic mass without cellular arrangement. This can be demonstrated by cross sections through single plasmodial strands at the light microscopical level (Pl. I 2). The tube-like protoplasmic strands are differentiated into a central endoplasm, surrounded by a peripheral ectoplasmic layer.

The more fluid endoplasm shows a characteristic shuttle streaming within the stationary gel-tube ectoplasm, altering its direction rhythmically. Finally, locomotion comes about by a somewhat longer streaming duration in one period determining in this way the direction of motion.

As complete plasmodia are unsuitable for the microscopical study of special problems, small pieces are used as models of the whole slime mold. The most frequently investigated models are (Fig. 1):

(a) isolated pieces of plasmodial strands cut out to desired length and thickness, which show normal shuttle streaming (Fig. 1a) and,

(b) protoplasmic drops which flow out after pricking the plasmodial strands with a fine needle and are resorbed again by the strands within one hour (Fig. 1 b).

The electron microscopical investigation of such protoplasmic drops by Wohlfarth-Bottermann (1962) proved the existence of fibrillar-like structures appearing during resorption in the peripheral ectoplasm of the drops. Figure 2 represents

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Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

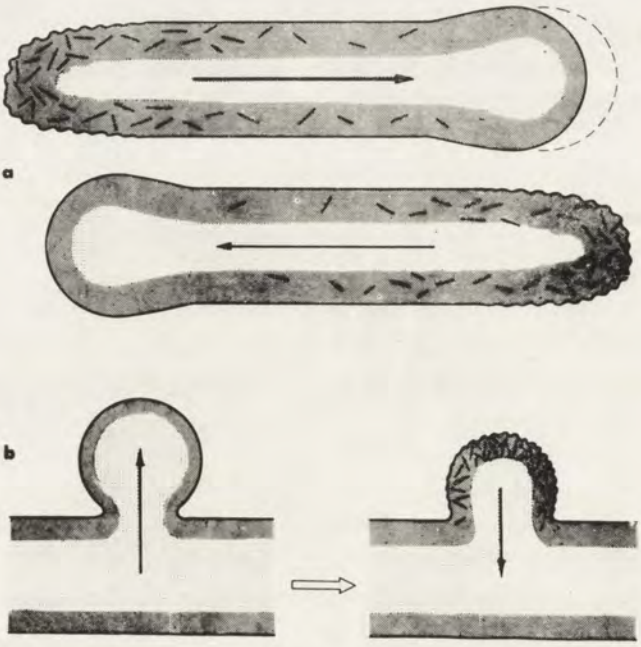

Fig. 1. a – isolated pieces of plasmodial strands of *Physarum polycephalum* showing ecto-endoplasm-differentiation and normal shuttle streaming caused by the formation and contraction of a system of fibrils, b – protoplasmic drops produced by lesion of plasmodial strands with a fine needle. The drops are resorbed again by the contraction of a fibrillar network, which is formed after 10 min in the peripheral ectoplasm

drawings of 3–5 μm thick paraffin sections cut through two protoplasmic drops of *Physarum polycephalum*, which were produced by lesion of a plasmodial strand. The upper drop (Fig. 2a) was fixed with OsO_4 just after it had emerged from the strand, the lower one (Fig. 2b) ten minutes later, when endoplasm begins to flow back again into the plasmodial strand. The difference between the two drops figured in this drawing elucidates that resorption is accompanied by the differentiation of numerous protoplasmic fibrils connected at nodal points with each other and thus forming a cohesive network passing through the peripheral ectoplasm of the drop. At the fine structural level (Pl. II 3) each fibril consists of many single filaments measuring 60–80 Å in thickness and is very similar to myofilaments found in smooth muscle. The plasmodial filaments are either random distributed without a preferential direction or orientated by parallelization. The arrows in the Pl. II 3 beside the fibril point to spindle-shaped, dense patches reminiscent of similar structures in smooth muscles. In interpreting the function of the protoplasmic fibrils Wohlfarth-Bottermann (1962, 1963, 1964 a, b, 1965) suggested the fibrillar network to be contractile in nature and hence responsible for the origin of hydrostatic pressure, causing the flow of protoplasm in the strands. In the last decade this has been supported by the experimental results from different laboratories throughout the world (Kamiya

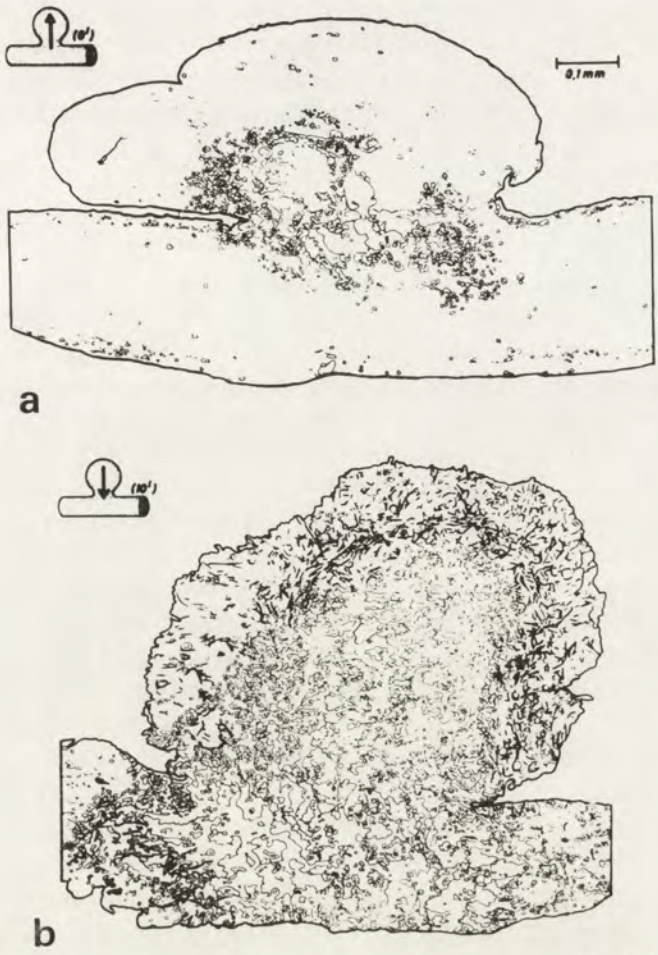

Fig. 2. Protoplasmic drop of *Physarum polycephalum*, which was produced by lesion of a plasmodial strand (*S*), a — fixed during efflux of endoplasm, b — fixed 10 min later, when the drop began to flow back. This "resorption" is brought about by contraction of the extensive fibrillar network in the peripheral ectoplasm. The resulting pressure causing backflow of protoplasm must behigher than that within the strand. Drawn from 3–5 μ thick paraffin sections. (From Wohlfarth-Bottermann 1964 b)

1959, Kamiya and Kuroda 1965, Hatano and Nakajima 1961, Hatano and Oosawa 1962, 1966 a, b, Nakajima 1964, Nakajima and Allen 1965, Ts'o et al. 1956 a, b).

These results are summarized below and completed by newer ones obtained mainly by investigations in our institute in Bonn. In order to give a clearer review of the present knowledge of motive force generation in slime molds, these results will be presented from three points of view:

(1) morphological observations concerning the changes and function of the fibrillar system;

(2) data resulting from comparative physiological and biochemical studies with other contractile systems using so called actomyosin-thread-models and,

(3) investigations carried out by combined biochemical and morphological methods in order to explain the macromolecular arrangement of the contractile structures.

Results

Morphology and function of the fibrillar system

Isolation of fibrils by simple methods developed by muscle physiologists enabled the proposed function of the fibrillar system in *Physarum polycephalum* to be rather easily tested. Kamiya and Kuroda (1965), Komnick et al. (1970) and Achterrath (1969 a and b) demonstrated that the protoplasmic fibrils survive for several days after glycerol extraction of either whole plasmodia or protoplasmic drops; thus they can be used as models as described by Hoffmann-Berling (1956, 1958). Plate III 4 (Achterrath 1969 b) shows a ten minutes old drop of slime mold protoplasm treated by the method of Kamiya and Kuroda (1965) for 10 days in a 50% glycerol-extraction-mixture before (Pl. III 4 a) and after (Pl. III 4 b) addition of a contraction solution. In the presence of ATP, K-, Mg- and Ca-ions the protoplasmic drop contracts to approximately 70% of its original volume. It is possible to inhibit this contraction by 1 mM Salyrgan, while the inhibition can be reversed by 30 mM cystein.

Electron microscopical investigations (Achterrath 1969 b, Komnick et al. 1970) of control and ATP-treated glycerol extracted protoplasmic drops made it evident that the contractile processes were accompanied by characteristic changes in the fibrillar fine structure (Pl. III 5). The filamentous network of the fibrils (Pl. III 5 a) became denser after addition of ATP (Pl. III 5 b). Except for this condensation, no other changes such as shortening or thickening of the filaments could be observed. These facts are similar to the circumstances described in smooth and striated muscles (Page 1964) and suggested that the plasmodial fibrils consist of actomyosin-like proteins which contract according to the sliding filament mechanism postulated by Huxley (1960). Since Ts'o et al. (1956 a, b), Loewy (1952) and Nakajima (1960) were able to isolate actomyosin-like proteins from slime mold plasmodia by KCl-extraction, the same method was employed in an attempt to remove the plasmodial fibrils. In order to prove the actomyosin-like nature of the fibrillar network and the responsibility of this system for the contraction process, glycerinated drops were treated with KCl-solution of high ionic strength and compared with controls (Komnick et al. 1970). The results obtained are presented in Pl. IV 6 and 7. Pl. IV 6 a–c shows a protoplasmic drop of *Physarum polycephalum* which was glycerol-extracted before ATP was added (a) and the same drop after addition of ATP (b); Pl. IV 6 c represents an outline drawing on transparent paper of the uncontracted state

(a) projected over the contracted one (b), illustrating in this way the degree of contraction. Pl. IV 6 d–f demonstrates the effect of KCl-treatment: this protoplasmic drop, which in addition to the glycerol bath had been extracted with KCl-solution of high ionic strength (d), has lost its contractile properties (e) as can be seen by the projected outlines (f). The morphological control of this experiment (Pl. IV 7) proves that the fibrils are lost by KCl-treatment: in comparison with the control (Pl. IV 7 a), which contains numerous thick plasmafibrils, the KCl-bathed protoplasmic drop shows empty regions very clearly indicating the former course of the fibrils (Pl. IV 7 b). This experiment supports the suggestion that the fibrils of *Physarum polycephalum* consist of an actomyosin-like protein whose contractile properties are responsible for the generation of motive force.

Investigations of actomyosin-thread-models

Further physiological and biochemical studies using so-called thread models (Weber 1935, Portzehl 1961) were begun in order to determine whether the structural organization of actomyosin-like protein, distributed either randomly in the groundplasm as single filaments or aggregated by parallelization to thick fibrils, plays a significant role in the contraction process (Beck et al. 1969, 1970 a, b). By this method it became possible to bring the isolated actomyosin of very different contractile systems to similar and comparable morphological levels. The isolation of actomyosin and preparation of thread models is summarized in Fig. 3 and

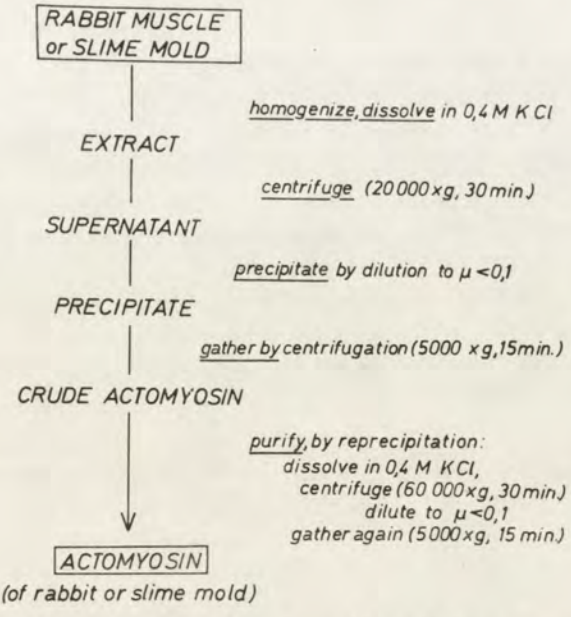

Fig. 3. Procedure for the isolation of actomyosin from different contractile systems

4. The threads measured approximately 5000 µm in thickness and attained a length of 1 m or more, if carefully handled. Little pieces – only a few mm long – were cut out and subsequently tested with ATP-containing solution. These pieces contracted, on an average, to 16.5% of their initial volume after the addition of 5 mM ATP. The corresponding linear contraction is 55%.

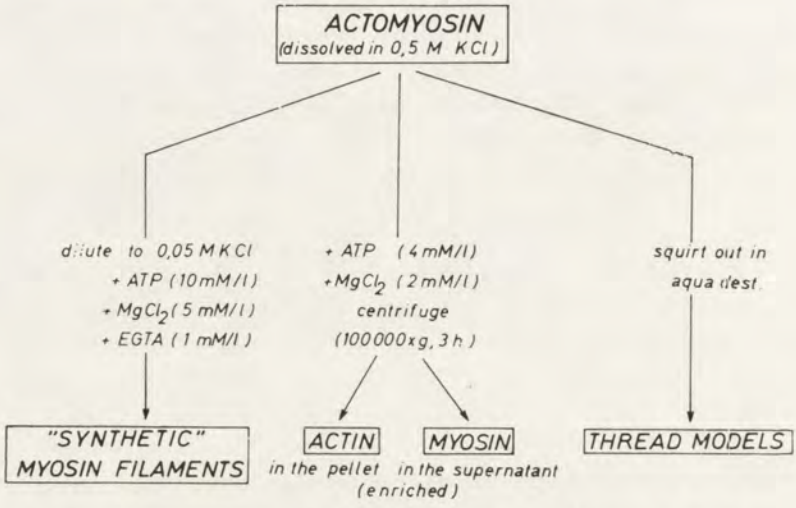

Fig. 4. Processing of isolated actomyosin by three different methods

In addition it can be demonstrated histochemically that both unfixed as well as formaldehyde-fixed threads show a positive reaction for ATPase activity. Using unfixed threads (Pl. V 8) the simultaneous demonstration of ATPase activity and contraction is possible. After adding 3.6 mM Pb^{++}, as a trapping ion, to the ATP-containing contraction solution and subsequent visualization with ammonium sulfide, the thread contracts and becomes black (Pl. V 8 a). The addition of 1 mM Salyrgan inhibits both ATPase activity and contraction (Pl. V 8 b).

From the study of ultrathin sections (Beck et al. 1970 a) of precipitated actomyosin threadmodels (Pl. VI 9) it can be concluded that the uncontracted (Pl. VI 9 a) and contracted threads (Pl. VI 9 b) from *Physarum polycephalum* show a fine structural similarity to natural motive plasmafibrils in corresponding states of contraction (compare Pl. III 5 a, b). They consist of a three-dimensional-network of loosely aggregated filaments. This network of unorientated filaments (Pl. VI 9 a) becomes dense after contraction without any increase in the filament thickness or decrease in the filament length (Pl. VI 9 b). These observations are further indications that the contractile proteins in the slime mold protoplasm contract according to a sliding filament mechanism.

Similar results can be obtained (Beck et al. 1969, 1970 b) with thread models of other contractile systems (Table 1). Actomyosin extracted with 0.6 M KCl from the

Table 1

Contraction of actomyosin-threads of different origin in ATP solution

Actomyosin threads	Average linear contraction (in %)	Distribution of the individual measurements (in %)
Foot of edible snail (*Helix pomatia*)	57	45–69
Chicken stomach	56	40–61
Skin-muscle-tube of earth worm (*Allolobophora terrestris*)	54	43–63
Skeletal muscle of rabbit	44	38–56
Plasmodium of *Physarum polycephalum*	55	51–60

smooth muscle of the snail foot and chicken gizzard, the obliquely-striated muscle of the earthworm body wall and the cross-striated skeletal muscle of the rabbit was precipitated in the manner previously described (Fig. 3 and 4). Irrespective of their origin these models all exhibit the same contraction behaviour with values ranging from 45% to 55% in linear contraction. Furthermore, they are all conspicuously similar in fine structure and density of their contractile substratum (Beck et al. 1970 b). Hence, it can be concluded that the differences in the structural organization of the contractile apparatus observed in situ do not have any significance for the fundamental process of contraction, and that contraction studied in the different muscles and in slime mold plasmodia occur by the same mechanism as apparently occurs in the models. The supposition that the structural organization has no significance in the process of contraction can also be proven in yet unpublished investigations of D'Haese in our institute. This author was able to stretch normal thread models from slime mold plasmodia and cross striated muscle (Pl. VII 10). Stretching the models up to 300% by well controlled tractive forces (Pl. VII 10 b) was followed by parallel orientation of the originally random distributed and unorientated filaments (Pl. VII 10 a). Subsequent addition of ATP-solution causes the stretched thread models to contract back to a normal value of around 55% in the linear way (Pl. VII 10 c). In consequence of filament orientation the stretched model loses its ability to contract in all directions, but not its contraction properties. Thus we may conclude that the contractile process is not dependent on a high structural organization. In our opinion orientation by means of filament-parallelization can be interpreted as the influence of tractive forces operating in one direction only. The comparison of stretched thread models from rabbit cross striated muscle (Pl. VIII 12) with natural plasmodial fibrils (Pl. VIII 11) exhibits the structural similarity of both systems best. One will agree that it is very difficult to distinguish between the objects. This illustrates that actomyosin isolated from cross striated muscle can be transformed

by precipitation into a contractile system very similar to the structural organization in protoplasmic fibrils. Furthermore, if the isolated actomyosin in orientated or non-orientated thread-models, irrespective of its orgin and structural organization, contracts according to the same mechanism as in the living system, there is strong evidence for the existence of a basic similarity between the contractile processes in primitive motile systems and different types of muscles on the macromolecular level.

Structure of contractile proteins

Recently scientists in different laboratories have engaged in studying the molecular arrangement of the actomyosin complex in amoebae and slime molds (Hatano et al. 1967, Nachmias and Ingram 1970, Pollard et al. 1970). Brief reference will be made to these results summarizing the most important fine structural aspects of slime mold actomyosin. As seen in Pl. IX 13 a actin filaments can be separated from purified extracts of plasmodial actomyosin (compare Fig. 4) and prepared for the electron microscope by the negative staining technique (Beck at al. 1970 a). The filaments consist of two twisted strands of globular G-actin measuring together 50–70 Å in thickness and showing a periodicity of 350 Å. A comparison of the molecular fine structure of plasmodial F-actin (Pl. IX 13 a) with that of muscle actin (Pl. IX 13 b) reveals no morphological difference between the two proteins. The same conformity in the molecular arrangement was frequently demonstrated by Hatano and Takahashi (in press) for plasmodial and muscular myosin in shadow casted suspension preparations (Pl. IX 14). The myosin molecules isolated from the cytoplasm of *Physarum polycephalum* show the typical head-tail configuration as described for muscular myosin and possess similar dimensions.

Furthermore the single plasmodial myosin molecules are able to aggregate in vitro by the influence of a so-called relaxation-solution (Hinssen 1970). Under these conditions (Pl. X 15) synthetic myosin filaments are built up consisting of a smooth middle segment measuring 0.18–0.25 µm and contain only tails of myosin molecules. This middle segment is limited at both ends by enlarged structures consisting of numerous heads of myosin molecules. Although such synthetic myosin filaments can easily be produced from actomyosin extractions (compare Fig. 4), morphological evidence for the existence of natural myosin filaments occurring in situ is still lacking. Nearly all filaments which were revealed in natural fibrils as well as in thread-models and normal ground-cytoplasm of *Physarum polycephalum* represent double-stranded F-actin. According to the present state of knowledge, under normal conditions in slime mold plasmodia only dimeric or oligomeric aggregates of myosin seem to interact with actin filaments. The contraction of this actomyosin-complex may occur by a modified sliding filament mechanism suggested previously by Panner and Honig (1967) for vertebrate smooth muscle contraction (Fig. 5). In contrast to the contraction mechanism of striated muscles (Fig. 5 a) postulated by Huxley (1960),

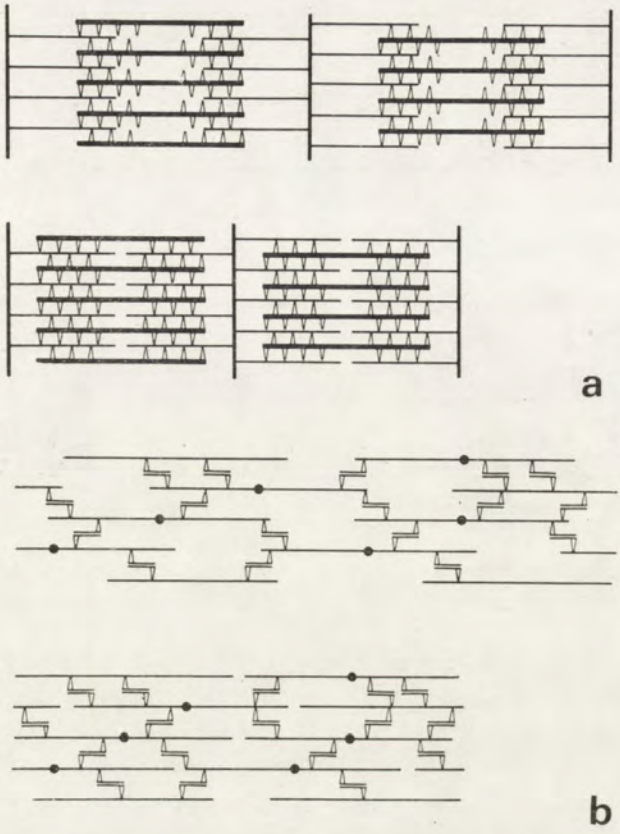

Fig. 5. Diagram of sliding filament mechanism a – of cross-striated muscle (after Huxley 1960) and b – of vertebrate smooth muscle (after Panner and Honig 1967). In both cases production of sliding movement occurs by tilting of cross bridges

Panner and Honig (1967) supposed that in smooth muscles the myosin is reduced to an oligomer state and that only actin is differentiated in a filamentous form (Fig. 5 b). The myosin molecules seem to operate as cross-bridges and sliding processes may occur between actin filaments only, which are connected with each other by the myosin molecules. In our opinion, this modified sliding filament mechanism may also occur in slime molds. Further investigations should prove this suggestion.

Concluding remarks

Finally, it may be summarized that recent results have elucidated the existence of a basic system of contractile proteins in the cytoplasm of acellular slime molds, which is responsible for the generation of motive force. The distribution of filaments and fibrils as well as physiological and biochemical results favour the hydraulic pres-

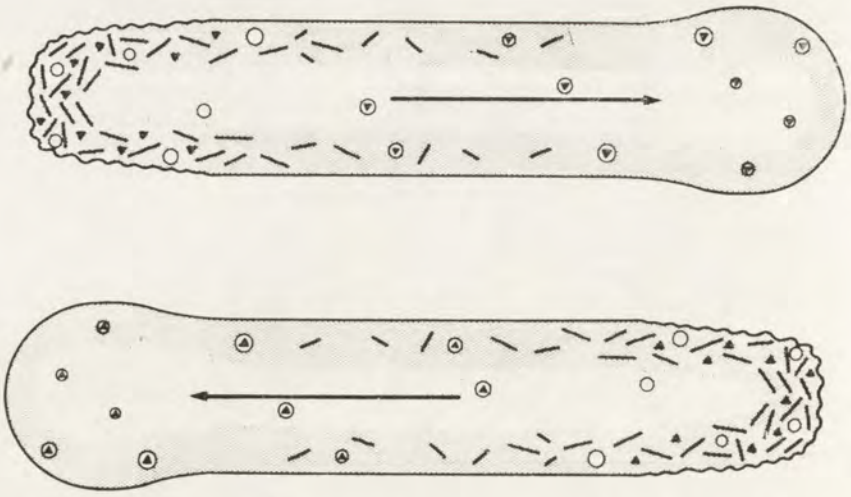

Fig. 6. Schematic representation of protoplasmic shuttle streaming in a plasmodial strand of *Physarum*. The rods indicate fibrils, which contract after proposed calcium release at the tail region. At the front region calcium is presumably accumulated within vacuoles. (From Komnick et al., in press)

sure flow theory (Fig. 6). As can be demonstrated by isolated pieces of plasmodial strands, protoplasmic streaming in *Physarum polycephalum* is due to differential pressure produced by the contractile properties of the ectoplasmic gel tube (Kamiya 1959, 1960, 1962, 1964). Filaments and fibrils in the outer zone cause the ectoplasm to contract and thus produce hyper pressure. Hence protoplasmic streaming results, alternating its direction rhythmically after a short period of streaming. This so-called shuttle streaming implies an extremely ephemeral character of the contractile structures and requires a mechanism controlling this phenomenon. In this connection it is of interest to note that Braatz and Komnick (1970) demonstrated the existence of a Ca^{++}-accumulating-system (Pl. XI 16) in slime mold plasmodia indicating that this cation may have a regulatory effect corresponding to that previously shown for muscular contraction. Similar to the L-system of skeletal muscle, plasmodia of *Physarum* accumulate Ca^{++} within certain vacuoles. This accumulation depends on the presence of ATP and is inhibited by salyrgan. Further investigations should prove the possible role of the calcium accumulating system in regulation of the motive force generation for protoplasmic shuttle streaming.

Furthermore, biochemical isolation and identification of the ATP sensitive proteins, actin and myosin, from slime mold plasmodia as well as the conditions required for contraction indicate that there is a very close relationship between plasmodial and muscular contractility. The mode of interaction between actin and myosin seems to be a sliding filament mechanism similar to that causing muscle contraction (Huxley 1960). However due to the lack of morphological evidence of natural myosin filaments in situ, contraction may occur by a modified sliding filament mechanism

suggested previously for vertebrate smooth muscle contraction (Panner and Honig 1967). The results, based on comparisons between thread models isolated from the different contractile systems, demonstrate that there is a basic similarity between the contractile mechanisms in primitive motile systems and muscle at the molecular and micellar levels.

Summary

To demonstrate the importance of amoeboid movement, the shuttle streaming of slime mold plasmodia is presented in detail.

The causal mechanism for protoplasmic movement has been found to be an ATP-sensitive system of contractile proteins within the cytoplasm, which morphologically appears as single filaments or fibrils. As in striated muscle, these filaments seem to interact by a sliding mechanism.

Actin and myosin from slime mold plasmodia were isolated and identified biochemically. Agreement with conditions in muscle contraction has been shown. In *Physarum polycephalum* actin filaments could easily be found, whereas demonstration of myosin filaments in situ has not yet been achieved. Production of synthetic myosin filaments, however is easily achieved. It is possible in slime mold plasmodia that a reaction of myosin in dimer or oligomer configuration with actin takes place, a mechanism which has already been suspected for vertebrate smooth muscle contraction.

The distribution of filaments and fibrils as well as physiological and biochemical results point to the existence of a hydraulic pressure flow mechanism which would imply an extremely ephemeral character of contractile structures.

In recent years the question of a control mechanism in shuttle streaming has been the subject of an increasing number of papers. The finding of a Ca^{++}-accumulating system in slime mold plasmodia points to a similar important involvement of this positively charged ion as was primarily found in muscle contraction.

Results obtained from slime mold plasmodia, as a representative for primitive motile systems, are compared with results found in the different types of muscles. Thread models of the acto-myosin complex obtained from the different contractile systems (acellular slime molds, smooth muscle and striated muscle) have been used for comparison to demonstrate the similarities of the basic contraction mechanism of primitive motile systems and muscles at the molecular and micellar level.

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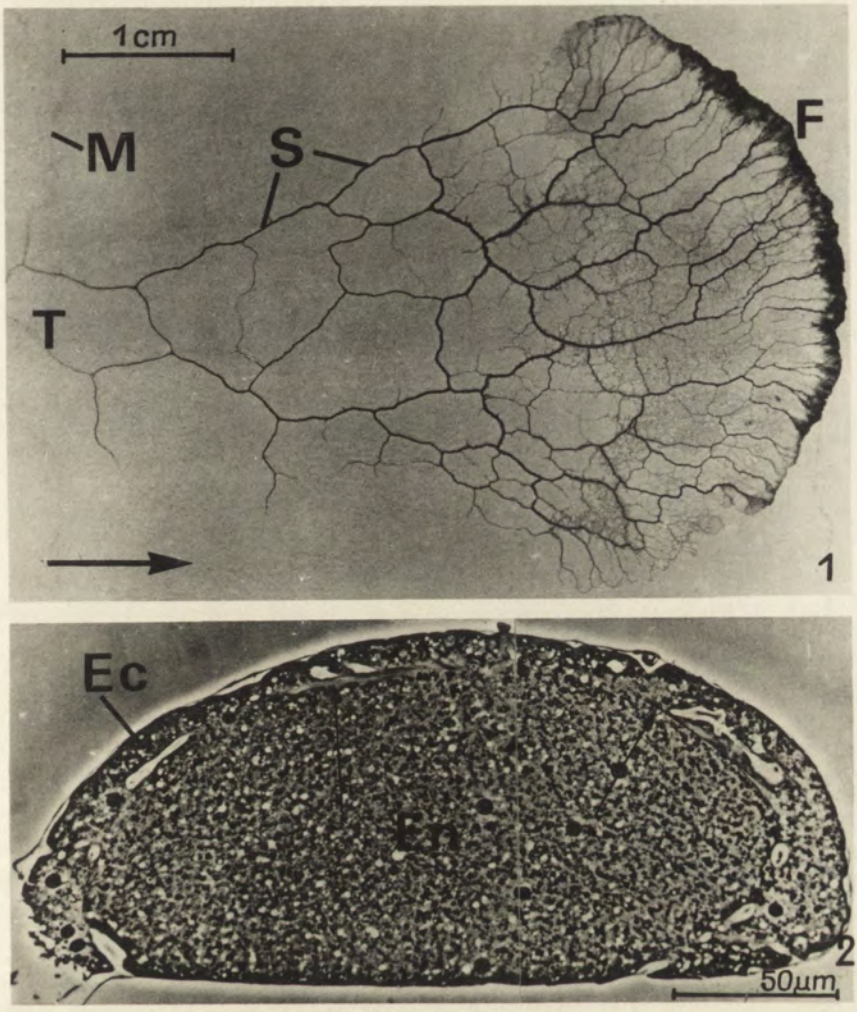
EXPLANATIONS OF PLATES I–XII

1: Form of a migrating plasmodium of *Physarum confertum*. Arrow indicates the direction of locomotion. F—front region; T—tail region; S—plasmodial strands; M—mucus trace left behind by the retracting strands. 2.2×
2: Cross section through a plasmodial strand. Ecto- and endoplasm are separated by a system of flattened membrane invaginations. En—central endoplasm; Ec—peripheral ectoplasmic layer. Arrows point to plasma fibrils. 470× (From Stiemerling 1970)
3: Branching plasmafibril of *Physarum polycephalum* consisting of parallel filaments approximately 80 Å thick. Arrows point to spindle-shaped dense patches. N-nucleus; V—vacuole; M—mitochondrion. 16 000× (From Wohlfarth-Bottermann 1965)
4: Ten minute-old glycerol-extracted protoplasmic drop before (a), and after (b) addition of an ATP-containing contraction solution. Contraction to approximately 70% of the original volume. 85× (From Achterrath 1969)
5: a—Fibril of an isolated protoplasmic drop of *Physarum polycephalum*, which had been bathed in DNP and thereafter extracted with cold 50% glycerol; b—the same from another drop which had contracted on the addition of ATP. 50 000× (From Komnick et al. 1970)
6: Glycerinated protoplasmic drops of *Physarum polycephalum*, which had been bathed with DNP for fibril multiplication. a—before ATP was added; b—the same drop after addition of ATP; c—outline of (a) drawn on transparent paper and projected over (b), showing degree of contraction; d—another drop which, in addition to that one in (a), had been extracted with KCl-solution of high ionic strength, before ATP was added; e—after addition of ATP; f—outline of (d) over (c) demonstrating that contractility was lost during extraction with KCl. 2.5× (From Komnick et al. 1970)
7: a—Section through isolated protoplasmic drop of *Physarum polycephalum* after 20 minutes, bath in 5.10^{-4} M DNP, showing many thick plasmafibrils; b—section through another drop which in addition had been extracted with 0.6 M KCl, showing that fibrils are lost, 1500×. Fig. 7 is the morphological control to the experiments shown in Fig. 6. (From Komnick et al. 1970)
8: Actomyosin thread model from *Physarum polycephalum*. a—simultaneous demonstration of ATPase activity and contraction by addition of 3.6 m M Pb^{++} as trapping ion to the ATP-containing "contraction solution" and subsequent visualization with ammonium sulfide; b—(con-

trol): Inhibition of ATPase activity and contraction by addition of 1 m M Salyrgan to the incubating solution. 50× (From Beck et al. 1970 a)
9: Ultrathin sections of an uncontracted (a), and contracted (b) actomyosin thread from *Physarum polycephalum* showing fine structural similarity to native plasmafibrils in corresponding states of contraction (compare with Pl. III 5). 150 000× (From Beck et al. 1970 a)
10: Ultrathin sections of non-orientated (a) and orientated (b and c) actomyosin threads of rabbit skeletal muscle. Thread stretched to 300% causes parallel orientation of the filaments. a—non-orientated, uncontracted; b—stretched and uncontracted; c—stretched, and after contraction by ATP. 50 000× (D'Haese, unpublished)
11: a—Plasmafibril of *Physarum polycephalum* consisting of parallel filaments about 80 Å thick. 50 000× (From Wohlfarth-Bottermann 1965)
12: Longitudinal section through a rabbit skeletal muscle—actomyosin-thread orientated by stretching, and contracted by ATP. The morphological similarity between (a) and (b) is significant. 50 000× (D'Haese, unpublished.)
13: a—Isolated and negatively-stained F-actin from *Physarum polycephalum*. 300 000× (From Beck et al. 1970 a); b—Diagram of muscle F-actin (Fom Huxley 1969)
14: Myosin molecules isolated from *Physarum polycephalum* and prepared by mica-replica-technique. (Fom Hatano, in press)
15: Synthetic myosin filaments from *Physarum polycephalum* which are formed by the influence of a "relaxation solution". a—90 000×; b—and c—200 000× (From Hinssen 1970); d—diagrammatic representation of the mode of aggregation of striated muscle myosin molecules to form filaments whose structural polarity reverses at the midpoint. (From Huxley 1963)
16: a—Calcium oxalate precipitate in vacuoles (arrows) of plasmodium of *Physarum confertum* resulting from experimental Ca^{++}-accumulation during incubation in 5 mM ATP, 5 mM $MgCl_2$, 4 mM $CaCl_2$, 4 mM EGTA, 5 mM $K_2Cr_2O_4$ and 20 mM histidine buffer, pH 7.0. 24 000×; b—enlarged calcium oxalate-containing vacuole. 46 000×; c—calcium oxalate precipitate within L-system of *Xenopus laevis* abdominal muscle incubated for control. (From Braatz and Komnick 1970)



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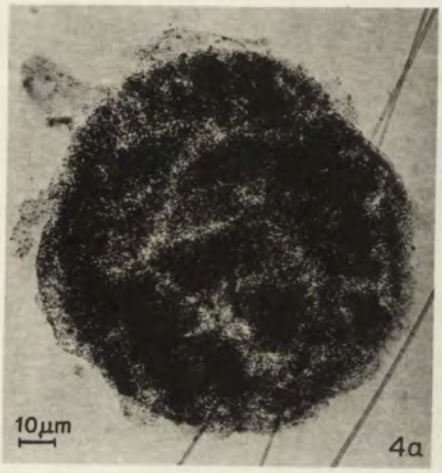

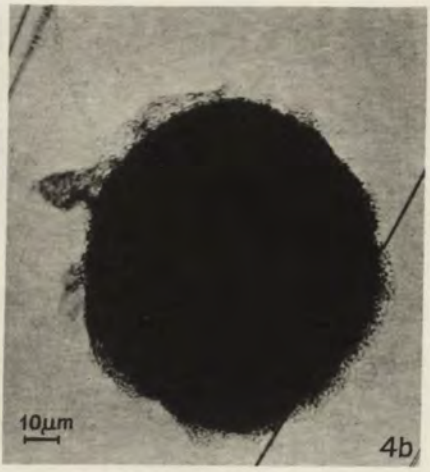

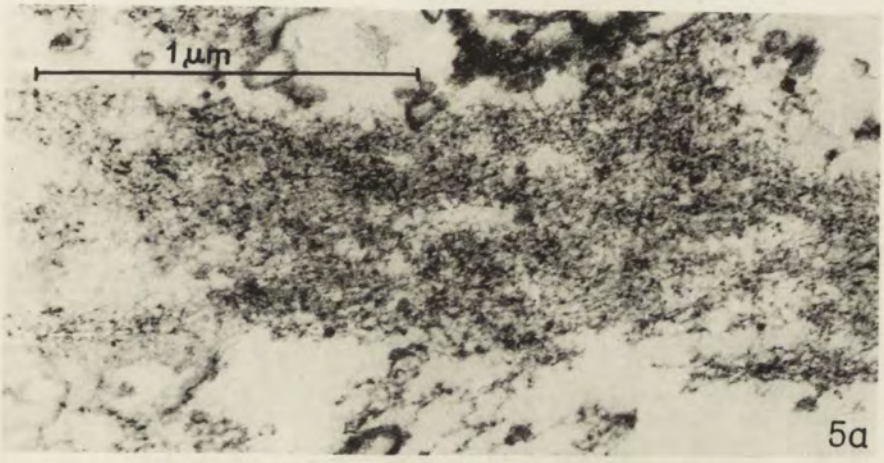

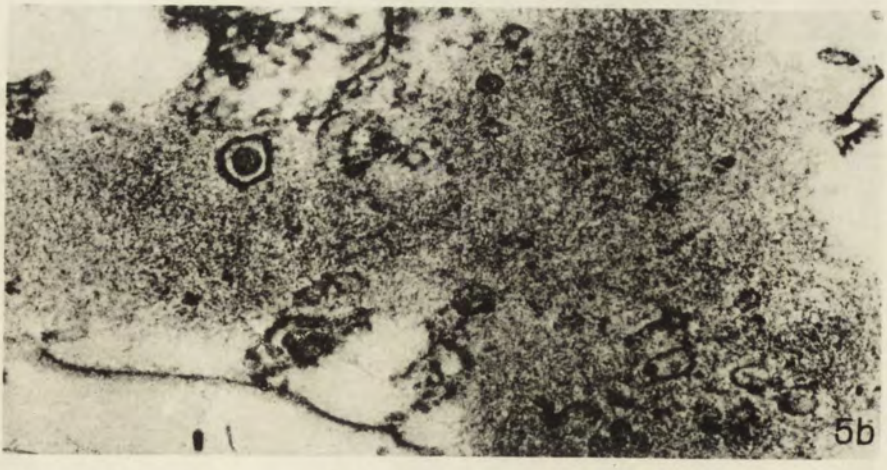

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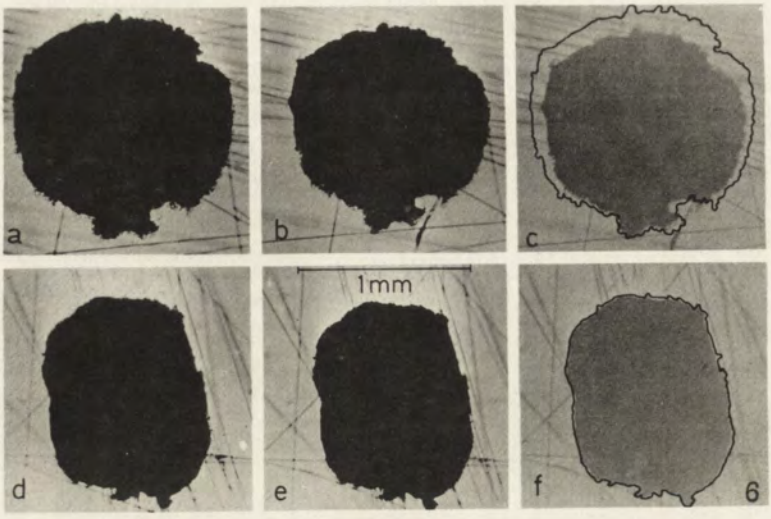

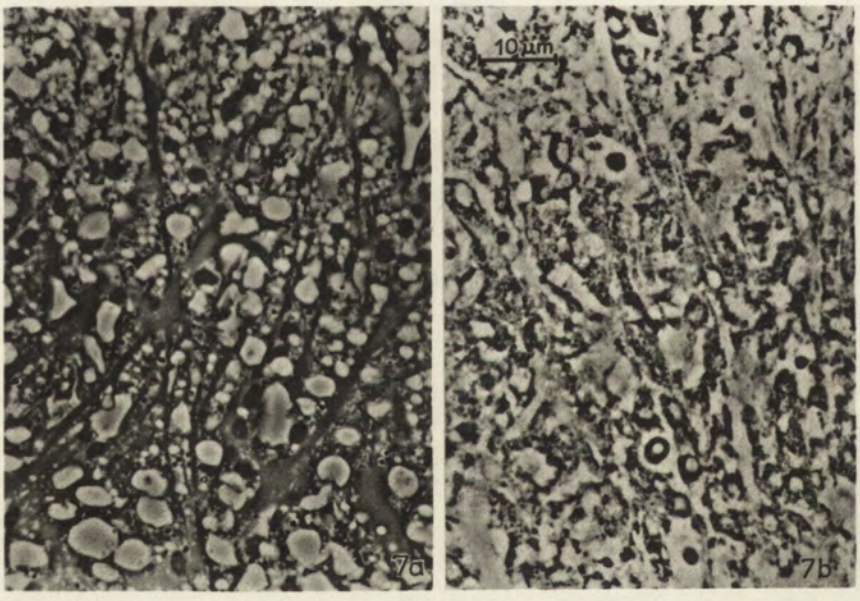

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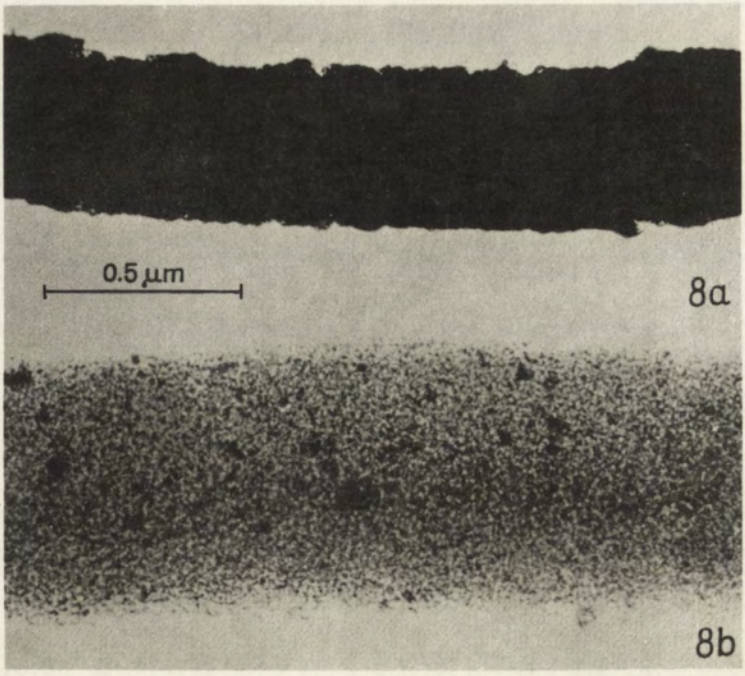

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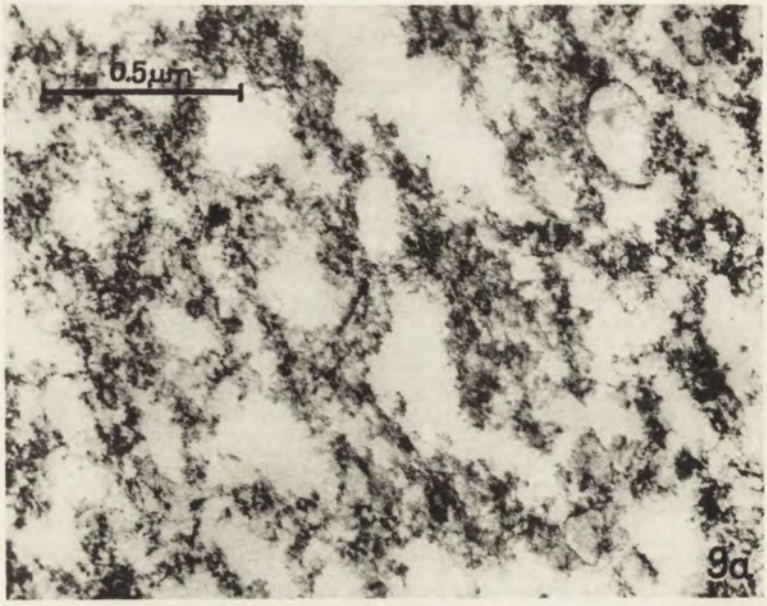

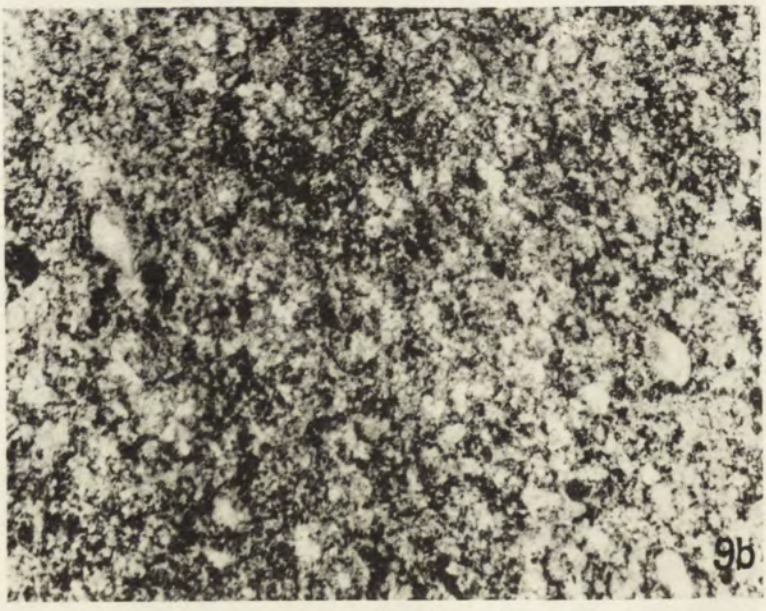

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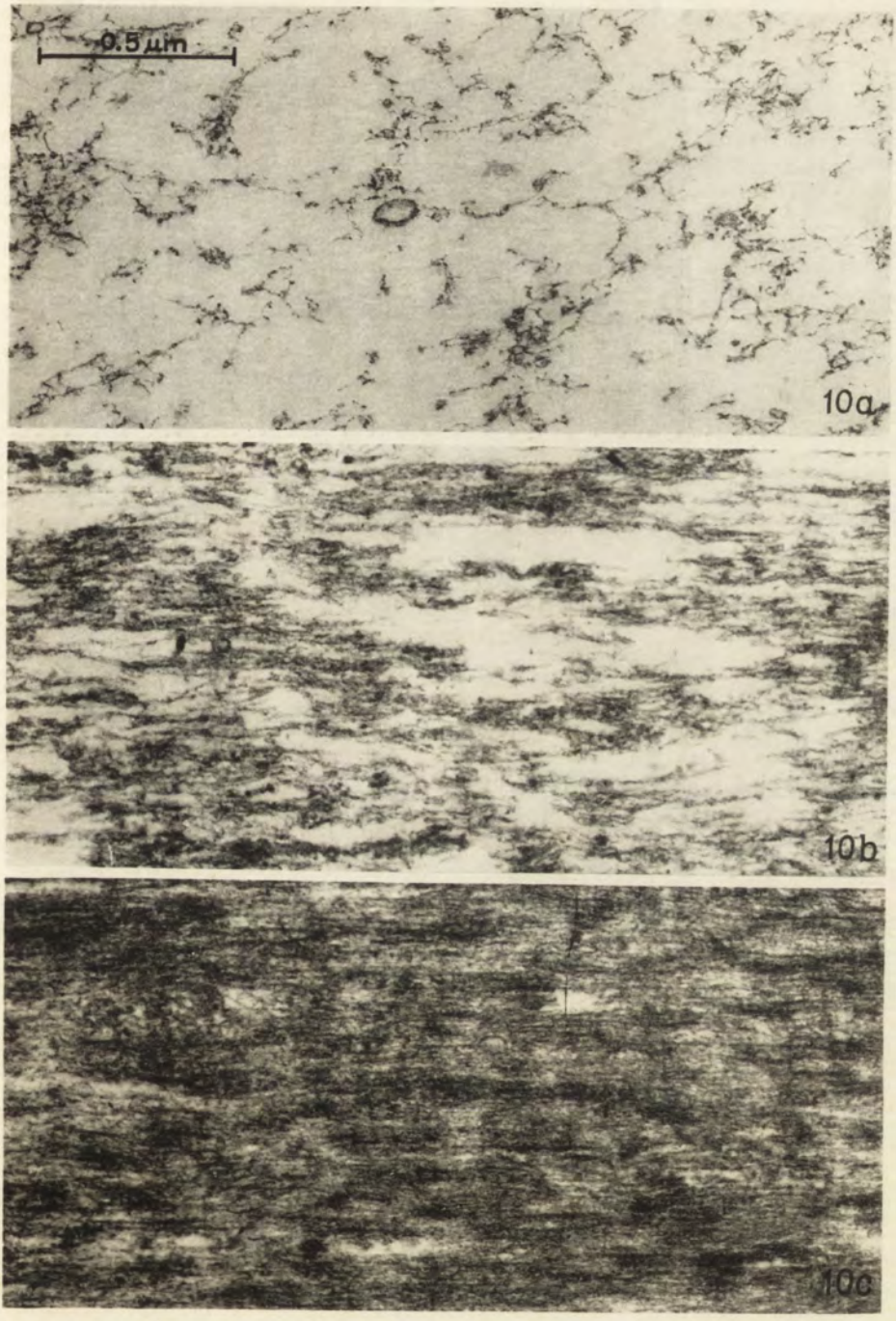

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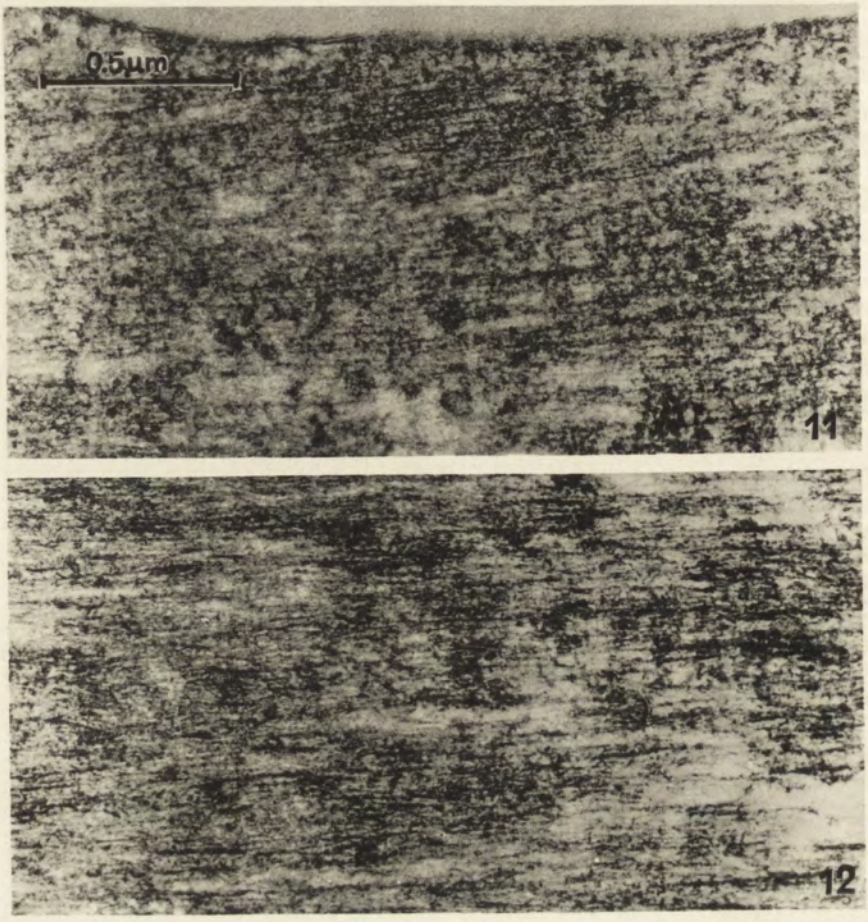

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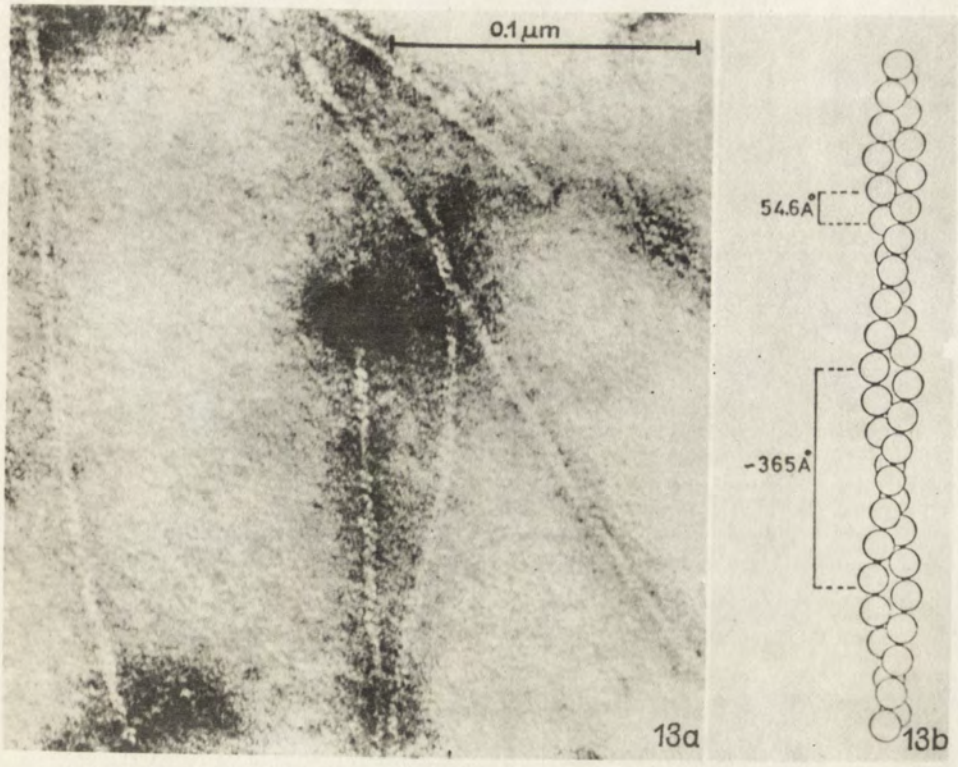

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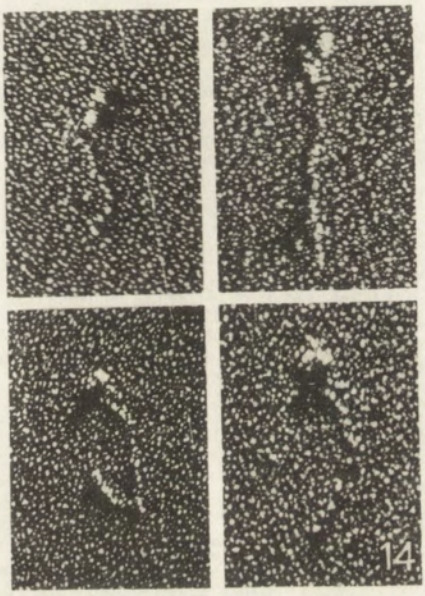

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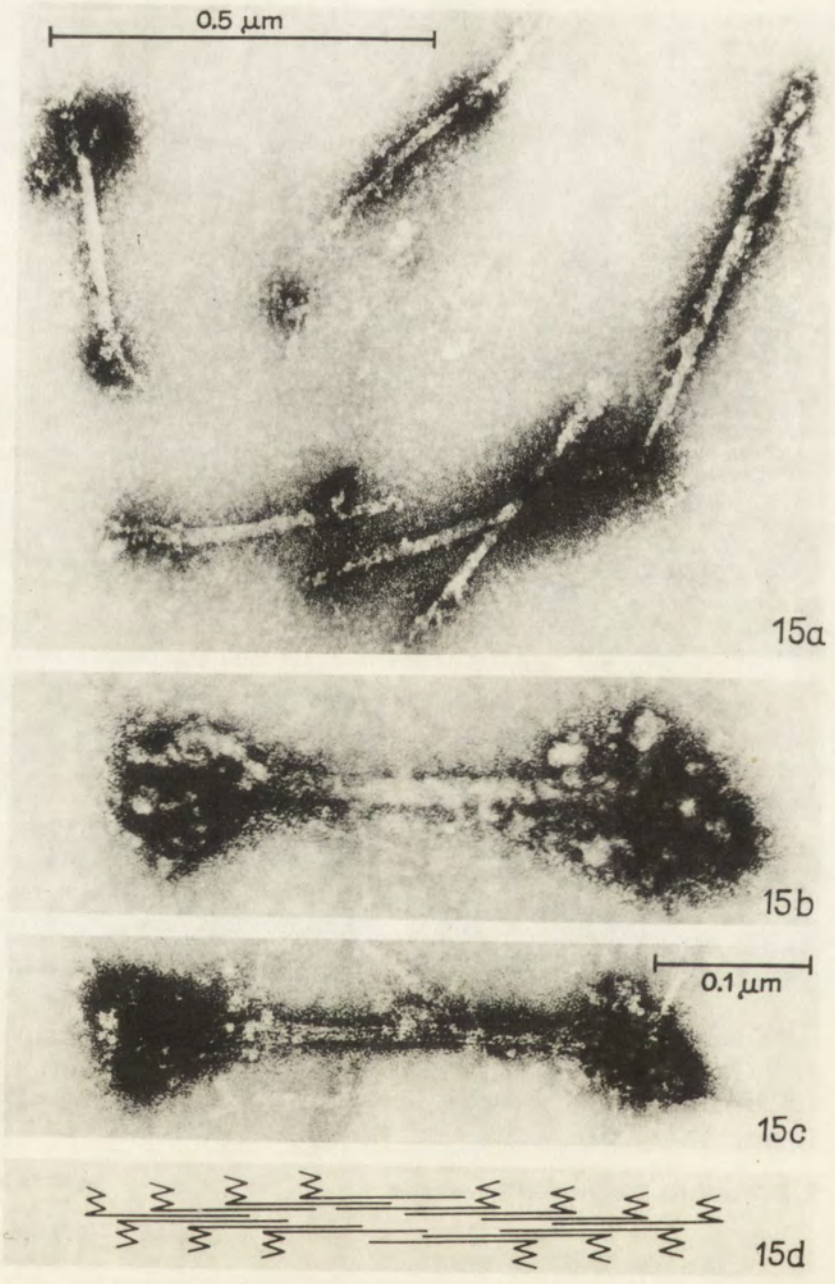

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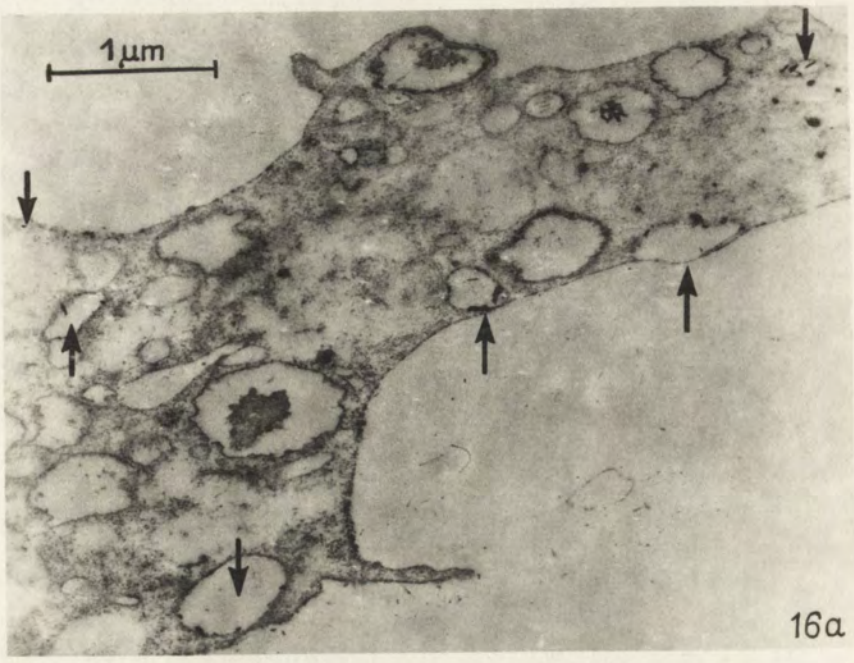

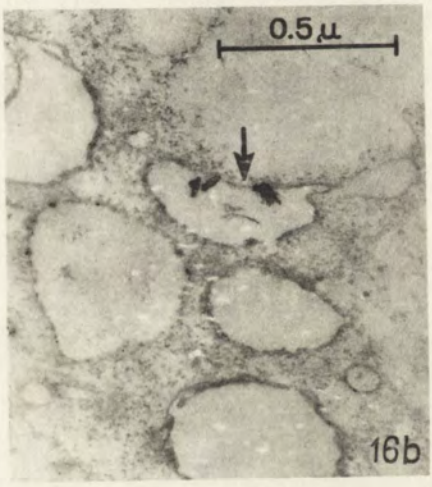

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ACTA PROTOZOOLOGICA

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Myosin from *Physarum polycephalum*

PPhysarum actomyosin prepared by the method of Hatano and Tazawa (1968) can l be used to prepare myosin-enriched actomyosin and free myosin as has been descrribed by Hatano and Ohnuma (1970). Using essentially the same method of hiighspeed centrifugation of the actomyosin in the presence of ATP and magnesiumn salts (Nachmias and Ingram 1970), we obtain an average of 4 to 5 mg free myossin from 100 grams of plasmodium. The Ca activated ATPase of the clear solutitions is 0.4 to 0.5 μMP_i/min/mg myosin and is markedly increased by 2-mercaptoethanol. 2-mercaptoethanol also antagonizes the inhibition of ATPase activity causeed by N-ethylmaleimide at a range of concentrations. We conclude that this myossin is a sulfhydryl containing enzyme. The *Physarum* myosin interacted with rabbbit actin as demonstrated by electron microscopy to form characteristic arrowheadd structures. Despite these similarities with striated muscle myosin, *Physarum* myossin remained soluble at low ionic strengths as has been previously reported. Onlyy after alkaline dialysis against 45% D_2O were aggregates formed which appeared like sshort filaments, but head-to-head aggregations also occurred. However, filaments 200 A° wide and 0.3 μ long were seen in some myosin-enriched actomyosin preparattions treated with relaxing solutions. It is concluded that evolutionary changes have taken place in the myosin tail region so that filament formation in this primitive myossin can occur as a result of actin-myosin interaction.

PPaper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

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Studies on motility in cytoplasmic extracts of *Amoeba proteus*

The study of amoeboid movement has been approached in several ways. Observation of living cells in the light microscope has revealed the wide diversity of movement patterns in different cell types and has defined the general properties of moving cells.

Recent biochemical studies of contractile proteins extracted from homogenates of slime molds and amoebae have established the presence of proteins related to muscle actin and myosin in these primitive cells, but it has been difficult to relate the properties of these purified "contractile" proteins to events in the moving cell (see Pollard, 1971 for a review).

A third approach, which is reviewed here, has been to study the properties of cytoplasmic extracts from amoebae which exhibit movement outside the living cell. This approach, which may eventually allow correlation of the biochemical data with observations on living cells, has several advantages:

(1) One can fractionate the extract, determine which fractions are necessary for movement, and then examine these essential fractions to determine which components generate movement.

(2) The absence of a plasma membrane barrier allows direct manipulation of the motile cytoplasm to study the factors controlling movement. It also allows easy fixation of the extract for electron microscopic observation.

(3) These experiments can be done with a few grams of cells, making possible experiments on the macromolecular level with cells that are difficult to culture such as the giant amoebae, *Amoeba proteus* and *Chaos carolinesis*.

These investigations began with the discovery by Allen, Cooledge and Hall (1960) that particles in cytoplasm freed from *Chaos* continue to move in nearly normal patterns. In 1963 Thompson and Wolpert reported a method for isolating a crude cytoplasmic extract from mass cultures of *Amoeba proteus* which exhibited dramatic movements when warmed to room temperature with ATP. By fractionating the extracts they showed that the plasma membrane was not essential for movement but that high speed centrifugation removed some component from the supernatant

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that was necessary for movement. Recombination of the supernatant and the pellet restored the ability to move (Wolpert et al. 1964). Electron micrographs of precipitates formed at the end of the movement showed thick "12 nm" wide filaments in the crude extract and thinner "9 nm" wide filaments in a more purified fraction. It was thought that the thinner filaments aggregated to form the thick filaments. Morgan et al. (1967) later identified even thinner (2–4 nm wide) filaments in high speed supernatants from these extracts.

Dr. S. Ito and I confirmed their observations on movement in these extracts and extended the electron microscopic observations. Our findings, detailed below, suggest that two types of filaments, resembling muscle actin and myosin, are involved with movement in the extracts (Pollard and Ito 1970).

Results and discussion

The crude cytoplasmic extracts are prepared by a slight modification of the method of Thompson and Wolpert (1963) and are then sealed under coverslips for observation in a phase contrast microscope. Movement requires warming to room temperature and is stimulated by added ATP. During the first few minutes, particles move in all directions, sometimes in streams with other particles. As in the living cell, the structures causing these movements are not resolved in the light microscope, but the viscosity of the extract increases during these movements, suggesting that some structural change has taken place. Electron microscopic examination of extracts fixed during movement show that numerous 7 nm wide filaments form in the extracts, accounting for the increase in viscosity. These thin filaments are closely associated with small clusters of 16 nm wide thick filaments.

After about 10 min, birefringent fibrils appear in the extract and grow in length and width. These fibrils, which consist of parallel arrays of the thin 7 nm filaments, appear to transmit the tension necessary to move particles in the extracts. These movements have been observed to continue up to 90 min.

Thin filaments

The 7 nm thin filaments have been identified tentatively as actin, because they form repeating arrowhead shaped ATP-dissociated complexes with muscle heavy meromyosin (Pollard and Korn 1971). These complexes along the thin filaments are indistinguishable from the complexes of heavy meromyosin with muscle, *Acanthamoeba* and *Physarum* F-actin. Morgan (1971) has recently studied fractions enriched in these filaments and found that they contain a protein with a molecular weight and sedimentation coefficient typical of actin.

Our morphological observations suggest that these thin filaments depolymerize in the cold. If this occurs in intact amoebae, it could explain the apparent drop in cytoplasmic consistency when amoebae are cooled.

Thick filaments

The thick filaments have not been studied biochemically, but they are morphologically similar to muscle myosin thick filaments synthesized in vitro (Huxley 1963). They are about 0.4–0.5 μm long and have tapered ends with terminal projections, bare central portions and filamentous substructure (Nachmias 1968; Pollard and Ito 1970; Holberton and Preston 1970). Recent observations on negatively stained preparations of thick filaments substantiates the impression gained from sectioned material, that the thick filaments have projections near their ends. Cross sections through this region suggest that these side arms may have 6-fold symmetry like those on muscle myosin thick filaments. This has been confirmed in preliminary analyses of those micrographs using the Markham rotation technique.

The recent studies of Holberton and Preston (1970) on glycerinated models of *Amoeba discoides* suggest that ATP stimulates the formation of these thick filaments by the aggregation of thin filamentous subunits. Our observations on the motile extracts of *Amoeba proteus* gave us the impression that the thick filaments were more stable than this, but it is possible that more thick filaments formed after ATP treatment. This question about the effect of ATP on thick filament stability will best be answered when purified preparations of thick filaments become available.

Function of the filaments

The function of these filaments has not been proven, but indirect evidence supports the idea that they are involved with movement in the extract, and by extrapolation, movement in the amoeba.

It is clear from our microscopic observations and those of Wolpert and his colleagues, that the bundles of thin filaments, which form as movement progresses, appear to transmit the tension to move particles in the extract. Particles do not seem to "crawl" along the filament bundles. Identification of these filaments as actin further supports a contractile role for these filaments, but it seems unlikely that actin alone could generate the force for these movements.

Two observations suggest that the thick filaments, which are suspected to be myosin on purely morphological grounds, are required for movement. (1). Centrifuging the crude extract at 10 000 g removes a pellet containing plasma membrane fragments, vesicles, mitochondria and thick filaments. The supernatant is non-motile. Warming it with ATP produces numerous thin filaments which aggregate as they do in the motile extracts. Thick filaments have not been observed in these non-motile supernatants. (2). The thin and thick filaments are meshed during active movement with the thin filaments radiating outward from the clusters of thick filaments. When movement stops, the thin and thick filaments are aggregated separately into large pseudocrystalline arrays having minimal contact with each other.

Can the hypothesis that the thin and thick filaments interact to generate the forces for amoeboid movement be related to observations on intact cells? It would

be desirable to study carefully the distribution of these filaments in an intact amoeba fixed while moving in a well defined manner, but it has been impossible to fix the giant amoebae without considerable alteration of their natural appearance. Thick and thin filaments corresponding to those seen in the extracts have been observed in several studies of intact amoebae, but their arrangement in a moving amoeba needs to be established. Such a study on the giant amoebae seems useless at the present time because of the lack of a suitable fixative; either improved fixatives or more cooperative amoebae are needed.

Conclusion

To date these motile cytoplasmic extracts have supplied some valuable information about the structures generating the forces for amoeboid movement, but continued investigation is needed to clarify the structure and function of the thick filaments, how the thick and thin filaments interact and how this interaction is controlled. Hopefully it will also be possible to study their arrangement within the cell to learn how their interaction might result in the movement of the cell.

Summary

Motile extracts of *Amoeba proteus* cytoplasm have been fractionated to determine which components are required for movement. The results are consistent with the postulate that thick filaments, resembling muscle myosin morphologically, and thin filaments, which have been tentatively identified as F-actin, must interact to cause movement.

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Identification of myosin in *Acanthamoeba castellanii* and human platelets

In an effort to explain the mechanism of cell motility at the molecular level, biochemists have searched for "contractile" proteins in many types of cells. Crude mixtures of proteins which share some properties with muscle actomyosin have been isolated from a number of non-muscle cells, but the heterogeneous nature of these preparations makes them difficult to analyze. Our goal has been to develop methods to prepare highly purified actin and myosin from primitive cells to allow the study of their chemical composition, physical properties, enzymatic activity and interactions with each other. Presumably detailed knowledge of these properties will explain how force for movement is generated in primitive cells.

Weihing and Korn (1969, 1970, 1971 a,b) have reported at this meeting and elsewhere on their work with *Acanthamoeba* actin. Other workers have carefully examined the properties of actin and myosin from the slime mold, *Physarum polycephalum* (Hatano and Tazawa 1968; Hatano and Oosawa 1966; Adelman and Taylor 1969 a,b; Nachmias and Ingram 1970; Hatano and Ohnuma 1970). In this paper we will summarize briefly our recent work on the myosins from *Acanthamoeba* (Pollard 1971) and human platelets (Adelstein and Kuehl 1971; Adelstein, Pollard and Kuehl 1971).

Acanthamoeba myosin

The identification of actin in the *Acanthamoeba* by Weihing and Korn (1969) suggested that the amoeba might also contain a myosin-like enzyme important for movement of the cell. We have identified such an enzyme in the cell, partially purified it and studied some of its important properties. This ATPase fulfills several reasonable criteria for being myosin, but differs markedly from muscle myosin in having a lower molecular weight and being soluble at low ionic strength.

Identification of myosin-like activity

Several preliminary observations suggested that the *Acanthamoeba* myosin might be different from skeletal muscle myosin: (1) In contrast to *Amoeba* proteus

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which does have thick filaments, the *Acanthamoeba* does not contain thick, myosin-like filaments (Bowers and Korn 1968). (2) Extracts of the amoeba in 0.9 M KCl do not exhibit the ATP-sensitive viscosity changes expected of actomyosin until muscle myosin is added (Weihing and Korn 1971 a). (3) It has been impossible to prepare typical actomyosin from *Acanthamoeba* by the procedures used for the preparation of muscle, slime mold and platelet actomyosin. (4) Gel filtration chromatograms of amoeba homogenates indicate the absence of any ATPase of the same size as muscle myosin or heavy meromyosin.

We began our search for *Acanthamoeba* myosin by examining the properties of the several peaks of ATPase activity obtained by gel filtration chromatography of amoeba homogenates. One ATPase peak, which eluted in the position expected for a protein of molecular weight of 100 000–150 000, has the properties expected of a myosin ATPase. At low ionic strength in the presence of Mg^{++} its activity was stimulated by added muscle F-actin and the enzyme bound to muscle F-actin and sedimented with it in the ultracentrifuge. The binding to actin was blocked by Mg-ATP.

Purification procedure

Cells are homogenized in 0.5 M KCl with pyrophosphate and dithiothreitol (DTT) at pH 7.0 and centrifuged at 100 000 g. The supernatant is then fractionated by step elution from DEAE cellulose, ammonium sulfate precipitation, gel filtration on Sephadex G-200 and chromatography on hydroxylapatite. The enzyme splits into two peaks, H-1 and H-2 during this last step.

Yield and purity

The enzyme accounts for about 0.1% of the cell's total protein and about 10% of the activity of the homogenate is recovered in the H-1 and H-2 fractions, the remainder having been lost during the purification. Neither of these final fractions is homogeneous, but in highly purified fractions up to 70% of the protein is accounted for by a single band on SDS-polyacrylamide gel electrophoresis. The specific activity is increased about 200 fold over the whole homogenate.

Enzymatic properties

H-1 and H-2 are identical enzymatically. The ATPase activity is highest in 0.5 M KCl with EDTA (2–3 μmole/P_i released min/mg protein). This EDTA–ATPase activity is dependent on K^+, like muscle myosin. Other characteristics of the amoeba ATPase also parallel those of muscle myosin: Ca-ATPase activity is about 10–20% of the K-EDTA activity and Mg^{++} inhibits the enzyme almost completely.

Identification as myosin

Two functional properties can be used to identify a protein as myosin: ability to form an ATP-dissociated complex with F-actin and ability to interact enzymatic-

ally with F-actin. Both H-1 and H-2 bind to muscle F-actin and sediment with it in the ultracentrifuge in the absence but not the presence of ATP. Also, actin activates this amoeba ATPase when assayed at low ionic strength in the presence of Mg. The magnitude of this activation increases during the first three steps in the purification procedure, in parallel with the purification of the enzyme, and has exceeded 40-fold activation. For unexplained reasons, the actin activation is markedly decreased in H-1, H-2 and recombined H-1 plus H-2.

Physical properties

Despite the close resemblance of the enzymatic properties of amoeba myosin and muscle myosin, there are striking differences between the amoeba and muscle myosins. Unlike muscle myosin, the amoeba enzyme is soluble, and does not aggregate, in 0.1 M KCl at pH 7.0 and the amoeba myosin is much smaller than muscle myosin. The major band on SDS polyacrylamide gel electrophoresis corresponds to a molecular weight of 140 000, which agrees with the size of the enzyme as determined by gel filtration.

Comments

The apparent low molecular weight of the amoeba myosin and its solubility at low ionic strength can explain the original difficulties in obtaining evidence for a myosin-like ATPase in the amoeba. We have been concerned, however, that we might have isolated only a fragment of a larger, native myosin molecule which may have been cleaved during extraction from the amoeba. To rule out this possibility, several control experiments have been done.

(1) Amoebas have been extracted with a variety of solutions which would solubilize muscle myosin and then fractionated on gel filtration columns. No high molecular weight K-EDTA ATPase which might correspond to a larger myosin molecule has been found.

(2) Prolonged extraction of the homogenate does not lower the yield of the enzyme or change its position of elution on gel filtration.

(3) There is little or no proteolytic activity in the homogenate at 0 °C as judged by the production of ninhydrin-reacting material.

(4) Inclusion of large amounts of the protease inhibitor di-isopropyl fluorophosphate (DFP) in the extracting solution does not alter the size of the K-EDTA–ATPase.

(5) The homogenate does not cleave muscle myosin into heavy meromyosin or subfragment-1 during a 90 min incubation, despite the fact that muscle myosin is very sensitive to attack by a variety of proteolytic enzymes.

Therefore, if the low molecular weight of the isolated amoeba myosin is the result of proteolytic cleavage, this cleavage must occur rapidly (even in the presence of DFP) and must convert quantitatively the hypothetical large amoeba myosin molecule into the smaller molecule by a reaction that does not attack the highly sensitive

muscle myosin molecule. Such a reaction has not been ruled out, but it seems unlikely.

The relation between H-1 and H-2, the two ATPase peaks eluted from hydroxylapatite is unresolved. At the present time, no important differences, aside from different contaminants, have been found between these two enzymes, so their separation may be artifactual. Further studies are in progress to answer this question.

Platelet myosin

The contraction of blood platelets which results in clot retraction is thought to be mediated by a complex mixture of proteins termed thrombosthenin. Thrombosthenin shares many properties with muscle actomyosin and can be separated into myosin-like and actin-like fractions (Bettex-Galland and Luscher 1965). The myosin component has been investigated by several laboratories who found that it is a high molecular weight Ca-ATPase with very low specific activity (Cohen et al. 1969, Booyse et al. 1971).

We have recently developed methods to fractionate human platelet thrombosthenin and discovered that it contains, in addition to actin and myosin, two components which presumably arise from the cleavage of the platelet myosin into its head and rod regions (Adelstein, Pollard and Kuehl 1971). Electron microscopic analysis of these fractions now gives us a detailed picture of the structure of platelet myosin and how it interacts with F-actin. The results suggest that the mechanism of contraction in platelets is closely related to that in striated muscle.

Purification procedure

Human platelet thrombosthenin is prepared by the method of Grette (1962) and then centrifuged at 100 000 g with Mg-ATP to remove part of the F-actin. The supernatant is then fractionated with ammonium sulfate. Intact myosin and myosin head are concentrated in the 30–50% saturation fraction, and almost pure myosin rod is found in the 50–60% fraction. The 30–50% fraction is then chromatographed on Sepharose 4B. Two peaks of ATPase activity are found: the first elutes at a position corresponding to a molecular weight of about 500 000 and consists of intact myosin slightly contaminated with myosin rod; and the second elutes at a position corresponding to a molecular weight of about 100 000 and consists of myosin head somewhat contaminated with platelet actin. The protein eluting between these two peaks is enriched in myosin rod.

Properties of the partially purified proteins

These partially purified proteins have been analyzed by SDS-polyacrylamide gel electrophoresis and tested for ATPase activity and ability to bind to muscle F-actin.

Intact platelet myosin has a subunit molecular weight of 200 000, and Ca^{++}

and EDTA-activated ATPase activities (about 0.6 μmoles/min/mg protein) and binds to muscle F-actin in the absence, but not the presence, of ATP.

Platelet myosin rod has a subunit molecular weight of 130 000 and little or no ATPase activity and does not bind to F-actin.

Platelet myosin head has a subunit molecular weight of about 100 000 and Ca^{++} and EDTA-activated ATPase activities and binds to muscle F-actin like intact platelet myosin.

These properties suggest that platelet myosin, myosin rod and myosin head are very similar to the corresponding muscle proteins: myosin, myosin rod and subfragment-1 (myosin head). The electron microscope observations described below support this hypothesis.

Electron microscopy

Platelet myosin is monomeric at high ionic strength, but aggregates at low ionic strength to form short tapered bipolar filaments with terminal projections. The length of these thick filaments does not exceed 0.7 μm, but in other respects they closely resemble longer thick filaments of muscle myosin formed in vitro (Huxley 1963). The projections on these thick filaments bind to muscle or platelet F-actin to form polarized arrowhead-shaped complexes typical of actomyosin from muscle. The bare central region of the platelet myosin thick filament is frequently seen bridging between two actin filaments to which the thick filament side arms are attached.

Myosin rod is also monomeric at high ionic strength but it rapidly aggregates into smooth tapered filaments at low ionic strength. In contrast to the platelet myosin thick filaments, these rod aggregates lack side arms and continue to grow in length and width while in low ionic strength solutions. Addition of KCl rapidly disaggregates the rod molecules.

Myosin head does not aggregate into filaments at high or low ionic strength, but it forms bypical arrowhead complexes with muscle or platelet F-actin, which are dissociated by Mg-ATP. In keeping with its low molecular weight, no extended tail is observed on these myosin heads bound to actin.

Comments

It is clear from these findings that the structure of platelet myosin closely resembles that of muscle myosin. The intact platelet myosin appears to consist of a rod region, which aggregates to form the backbone of the thick filaments, and a head region, which projects from the backbone of the thick filament. The head region contains both the ATPase and actin binding sites. The platelet myosin thick filaments form complexes with F-actin which are essentiallly identical to complexes of muscle actin and myosin. These similarities suggest that the molecular mechanism of contraction in platelets is closely related to that in striated muscle.

The mechanism of the apparent splitting of intact myosin into its rod and head regions is not known. It may occur artifactually during the storage of the platelets

or during the purification procedure, or it may occur physiologically before the platelets are removed from the donors. These questions are under investigation, but the fact that this cleavage occurs has been of great value in analyzing the structure of the platelet myosin.

Discussion

Some of the properties of myosin isolated from striated muscle, *Acanthamoeba*, human platelets and *Physarum* are summarized in Table 1. As one might predict, the myosins from the two vertebrate sources are the most similar, but all of the myosins share three important properties: inhibition of the ATPase by Mg^{++}, activation of the Mg-ATPase by actin, and ability to bind to F-actin. These three properties are essential for contraction in striated muscle and their presence in more primitive motile systems suggests that they have a central role in the contractile process.

Table 1

Properties of several myosins

Source of myosin	Actin binding	Actin activation	ATPase			Molecular weight	Solubility and form at low μ
			K-EDTA	Ca^{++}	Mg^{++}		
Rabbit skeletal muscle[a]	yes	+++	++	+	−	460 00	−(thick filaments)
Acanthamoeba	yes	++	++	+	−	140 000[b]	+(monomers)
Human platelets	yes	+	+	+	−	450 000[b]	−(thick filaments)
Physarum[c]	yes	+	−	++	−	460 000	+(?dimers)

[a] See Huxley 1969, for a review.
[b] Estimated by gel filtration.
[c] Adelman and Taylor (1969); Hatani and Ohnuma (1970); Hatano and Tazawa (1968).

In resting striated muscle the myosin ATPase is strongly inhibited by the high intracellular concentration of Mg^{++}. During contraction, this Mg-ATPase activity is strongly activated by the interaction of myosin with F-actin. The energy released by ATP hydrolysis is somehow converted into the mechanical energy which causes the actin and myosin filaments to slide by each other (see Huxley 1969, for a review). For this system to work, the myosin ATPase must be inhibited by Mg^{++} and the myosin must be able to interact mechanically and enzymatically with F-actin. Since these properties are shared by the non-muscle myosins, the molecular mechanism of contraction may be similar in each of these cells.

Although similar, the myosins also clearly differ from each other in several ways. Some of these properties, such as the ATPase activity in high concentrations of KCl or Ca^{++}, are unimportant physiologically. Other differences may be related to the way the contractile system is organized within the cells. For example, both striated muscle (Huxley 1963) and platelets (Zucker-Franklin 1971) have thick filaments corresponding to myosin, but neither *Acanthamoeba* nor *Physarum* have such thick filaments. Correspondingly, the myosins from muscle and platelets aggregate into filaments at low (physiological) ionic strength, while the myosins from *Acanthamoeba* and *Physarum* do not. Presumably there is some functional significance to these differences, although it is not presently understood.

Summary

Myosin-like enzymes have been isolated from *Acanthamoeba* and human platelets. The *Acanthamoeba* myosin closely resembles muscle myosin in enzymatic properties and ability to bind to and interact enzymaticaly with muscle F-actin, but differs from muscle myosin in having a low molecular weight and being soluble at low ionic strength. Platelet myosin resembles muscle myosin even more closely, having similar enzymatic activities and structure; this similarity is supported by the identification of two fragments of the platelet myosin, and which correspond in structure and function to subfragment-1 and myosin rod from muscle myosin.

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Isolation and properties of actin from a small soil amoeba, *Acanthamoeba castellanii*

Actin and myosin appear to be present in motile systems besides muscle[1], and are undoubtedly present in a very actively motile organism the acellular slime mold, *Physarum polycephalum* (Adelman and Taylor 1969, Hatano and Ohnuma 1970, Hinssen 1970, Nachmias et al. 1970, Nachmias and Ingram 1970). Because the physical and enzymatic interaction of actin and myosin from skeletal muscle can be easily related to contraction in this tissue (Huxley 1969), it is natural to postulate that actin and myosin interact to cause movement in *Physarum* and other non-muscle cells in which they occur.

In 1968, Bowers and Korn reported that the cytoplasm of *Acanthamoeba castellanii*, a soil amoeba which crawls rather sluggishly, but which carries out active phagocytosis (Weisman and Korn 1967), contains numerous filaments of indefinite length and about 5–7 nm wide. These dimensions are like those of the thin actin filaments of muscle, so it seemed reasonable to try to isolate actin from the amoeba. In this paper we outline experiments which show: (1) that *Acanthamoeba* contains actin that closely resembles actin from rabbit skeletal muscle and (2) that the cytoplasmic filaments of *Acanthamoeba* can be identified with actin. We then briefly outline the problems that must be solved in order to specify exactly the role of the cytoplasmic actin filaments in movement of the amoeba.

Results

Actin was identified in amoebas in the following way. (For details see Fig. 1 of Weihing and Korn 1971 a.) Amoebas were extracted in 0.93 M KCl, pH 7.5 and the mitochondria and unbroken cells were removed by centrifugation. The

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[1] Pollard (1971 b) provides a critical discussion of certain claims that actin or myosin are present in non-muscle cells. Jahn and Bovee (1969) provide an encyclopedic list of motile cells which may contain "actinoid" and "myosinoid" proteins.

extracts were then tested for ATP sensitivity. If actin and myosin were present in the extract then we expected that the viscosity of the extract would fall upon addition of ATP owing to the dissociation of actin and myosin by the ATP. Only small, poorly reproducible viscosity changes were observed. This indicated that if actin and myosin were present in the amoeba, that one or both components were present in very low concentration. Since it was very easy to see actin-like filaments in the intact amoeba, we postulated that actin was present in suitable concentration, but that myosin was either present in low concentration or in some unusual form. We therefore added highly purified muscle myosin to the extract and found that the final viscosity was higher than the sum of the individual viscosities of the myosin and the amoeba extract alone. This was the result expected if the added myosin had combined with an actin-like component of the extract. Addition of ATP caused the viscosity to fall to a value close to that of the sum of the viscosities of the extract and myosin. This showed that the ATP had dissociated the myosin from the presumed actin component. After a few minutes the viscosity recovered, presumably owing to hydrolysis of the ATP by the myosin ATPase and cellular ATPases.

The actin identified in this indirect manner was isolated in the following way. (For details see Weihing and Korn 1971 a.) Highly purified muscle myosin was added to the amoeba extract and the amoeba actin was precipitated as a hybrid complex of actin and myosin by lowering the ionic strength down from 0.93 M KCl to 0.05 M KCl by dialysis. After washing the precipitate free of salt, it was dried with acetone which denatures the myosin. The actin was then extracted from the powder with a solution of 5×10^{-4} M ATP, 3×10^{-3} M cysteine, pH 8. The actin was concentrated by isoelectric precipitation at pH 5. After dialysis of the actin versus the ATP-cysteine buffer, the actin was polymerized with 0.1 M KCl, the polymers were collected by preparative ultracentrifugation, and the actin was depolymerized by dialysis versus the ATP-cysteine buffer. Final purification was achieved by gel filtration on Sephadex G-200 in ATP-cysteine.

Purification of the actin was measured in two ways. The reduced viscosity of the F-actin which could be formed at each stage of purification increased continually during the isolation and reached a final value of about 3 dl/gm. The content of 3-methylhistidine, an unusual amino acid which is present in every other actin in which it has been searched for (Johnson et al. 1967, Woolley 1970) and of ε-N-dimethyllysine (an unusual amino acid which is present in *Acanthamoeba* actin (Weihing and Korn 1970) but not rabbit actin (Kuehl and Adelstein 1969)) also increased during the purification to reach a final value of about one mole each per mole of protein. (For details see Table I, Weihing and Korn 1971 a.)

The purified amoeba actin was found to be highly homogeneous as judged by equilibrium ultracentrifugation and polyacrylamide gel electrophoresis. Purified amoeba actin, dissolved in 5 M guanidine hydrochloride and 1 mM 2-mercaptoethanol, was centrifuged to equilibrium. The plots of the logarithm of protein concentration versus the square of the distance from the center of the rotor were linear

at several different speeds, indicating that the actin was highly homogeneous. (For details see Fig. 7, Weihing and Korn 1971 a.) Electrophoresis of reduced and alkylated actin on polyacrylamide gels containing 8.5 M urea showed that both muscle and amoeba actin migrated as single major bands with very minor bands inconsistently present. (For details see Fig. 8, Weihing and Korn 1971 a.) These two criteria showed that the amoeba actin was probably more than 90% pure.

The molecular weight of amoeba actin was calculated from the slopes of the plots derived from the equilibrium centrifugations and from the partial specific volume calculated from the amino acid composition. The molecular weight found was 39 500. This is slightly smaller than the currently accepted molecular weight of muscle actin, which is 46 000 (Rees and Young 1967). We are currently investigating whether this difference is also observed in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The polyacrylamide gel electrophoresis in urea showed that amoeba and muscle actins migrate identically. This shows that the net charges on the two proteins are the same, which is consistent with the near identity of the amino acid composition of amoeba and muscle actins.

The purified protein was identified as actin by several different criteria. The first of these was its ability to exist as a monomer at the low ionic strength of the ATP-cysteine buffer and as a filamentous polymer when the ionic strength was raised to 0.1 M KCl. At low ionic strength the protein sediments as a single component with sedimentation coefficient of 3S. This sedimentation coefficient is consistent with a monomer molecular weight of 39 500. In 0.1 M KCl the 3S monomer is converted into a 30S polymer. The 30S boundary shows marked self sharpening suggesting that the polymer is highly asymmetrical. (For details see Fig. 3, Weihing and Korn 1971 a.) Amoeba actin polymerized with 0.1 M KCl exhibits flow birefringence, which showed that the actin polymers are highly asymetrical, thus confirming the deduction made from the ultracentrifugal patterns.

Amoeba actin filaments were identified directly by negative staining electron microscopy. (For details see Plate I. Pollard et al. 1970.) The filaments were about 6 nm wide and showed the 37 nm periodicity expected from previous studies on muscle actin.

The second criterion used to identify the amoeba actin was its ability to interact with rabbit muscle heavy meromyosin. The meromyosin formed arrowhead shaped structures along the actin filament which all pointed in the same direction and which were regularly spaced 37 nm apart. Decorated amoeba actin and rabbit actin filaments could not be distinguished. Moore et al. 1970 have shown that precisely spaced and oriented arrowheads are seen because each subunit of the double helical actin filament binds heavy meromyosin in a precise orientation which is repeated at every half pitch of 37 nm. Observation of arrowheads with amoeba actin, therefore, provides strong evidence that amoeba actin is a double helical filament.

The third criterion used to identify the amoeba actin was its ability to activate

the Mg-ATPase of rabbit muscle heavy meromyosin. The ATP-ase was measured by the pH-stat method of Eisenberg and Moos (1967). (For details see Table IV, Weihing and Korn 1971 a.) The Mg-ATPase activity of rabbit heavy meromyosin alone is very low, about 0.03 μmole Pi/min/mg. When rabbit actin was added, but all other conditions held the same, the ATPase was activated 22 fold. When the same concentration of amoeba actin was substituted for muscle actin a five fold activation was observed. The activation by amoeba actin was inversely proportional to the ionic strength but was directly proportional to the actin concentration. All these effects have previously been observed for muscle actin (Eisenberg and Moos 1968). Amoeba actin and muscle actin therefore interact in the same way with heavy meromyosin.

The interaction of amoeba actin with heavy meromyosin can be controlled by native tropomyosin from skeletal muscle in the same manner as the interaction of muscle actin with heavy meromyosin. Thus, in the absence of Ca, native tropomyosin prevents the interaction of amoeba actin with heavy meromyosin and the Mg-ATPase is not activated. Addition of Ca ion reverses this inhibition by native tropomyosin, and amoeba actin will then activate the Mg-ATPase about 10 fold. (For details, see Eisenberg and Weihing 1970.) Since native tropomyosin does not interact with heavy meromyosin, we must conclude that amoeba actin contains sites which interact with native tropomyosin from rabbit muscle. These results suggest that control of contractility in the amoeba and in skeletal muscle may operate in the same way.

The above experiments which show that both heavy meromyosin and native tropomyosin interact with amoeba actin in the same manner as with muscle actin suggest that the interacting sites on the actins are similar. This in turn suggests that the amino acid sequence and hence the three dimensional conformations of the two proteins are very similar. Proof of this postulate must await complete sequencing or X-ray diffraction analysis of the two proteins. However, we have found that the amino acid composition of the amoeba actin is very similar to that of rabbit actin. This extends to the presence of the unusual amino acid, *3*-methylhistidine, which is present in both actins. (For details see Table III, Weihing and Korn 1971 a.) This similarity in composition is consistent with, but does not prove the postulate that the various active sites of amoeba and muscle actin are very similar.

We have compared the composition of that portion of the amoeba actin molecule which contains *3*-methylhistidine with the corresponding portion from rabbit actin. (Weihing 1971 and Weihing and Korn 1972.) Amoeba actin was cleaved with cyanogen bromide and the peptide containing *3*-methylhistidine was isolated by gel filtration and DEAE cellulose chromatography as previously published for rabbit actin. (Adelstein and Kuehl 1970, Elzinga 1970). The overall composition of the amoeba peptide was very similar to that of peptide CB-10 from muscle actin. In particular the peptide contains one mole of *3*-methylhistidine per mole of peptide as expected from the composition of the muscle peptide. Also as in the muscle peptide,

the content of histidine and phenylalanine is nearly zero, but the peptide contains one or two of the five tryptophans present in amoeba actin.

The composition of two other amoeba peptides has been examined and is quite close to that of two other muscle actin peptides. One of these is the amoeba peptide which contains ε-N-dimethyllysine. The amoeba peptide does not differ significantly from muscle actin peptide CB-16 except for the presence of the methylated lysine. A third amoeba peptide is rich in glutamic acid but contains no proline. This peptide closely resembles peptide CB-17 from muscle actin.

These three peptides together account for 111 out of about 400 amino acids present in actin or about one fourth of the protein. Their similarity to the corresponding muscle peptides suggests, but does not prove, that these regions of the molecule have identical sequences. It will be recalled however, that the molecular weight of amoeba actin is about 6000 daltons less than the molecular weight of muscle actin. If this difference can be confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, then we will have to conclude that other regions of the two molecules must be different.

What is the relation of this actin, which so closely resembles skeletal muscle actin, to the cytoplasmic filaments of *Acanthamoeba*? To answer this question, we have shown that the intracellular filaments in glycerinated *Acanthamoeba* can be decorated with heavy meromyosin and that the formation of the complex can be inhibited by addition of ATP. In addition, the spacing of the arrowheads is about 35 nm which is close to the value of 37 nm found for the purified actin. (For details see Plates IV, V, and VI of Pollard et al. 1970.) Thus the intracellular filaments behave exactly as the purified actin does, and we have therefore concluded that the intracellular filaments are actin.

Does the presence of actin in *Acanthamoeba* imply the presence of myosin? It will be recalled that amoeba extracts did not show ATP sensitivity. Since actin is undoubtedly an important component of the amoeba cytoplasm this must mean that myosin is not present or that it is a very unusual myosin and Pollard (1971 a); Pollard et al. (1972) have reported experiments which suggest that *Acanthamoeba* contains an unusual myosin of low molecular weight. The *Acanthamoeba* protein is an ATPase which is activated by Ca and EDTA and inhibited by Mg. It forms complexes with muscle actin, the formation of the complexes is inhibited by ATP, and the Mg-ATPase is activated by actin. By these criteria the molecule is undoubtedly myosin. However, the molecular weight is about 140 000 or about 30% of that of normal myosin, and the molecule does not precipitate at low ionic strength. Using these criteria, many workers would doubt whether the molecule really is myosin, or they would suggest that the molecule is really a proteolytic digestion product of a more normal myosin. Suitable control experiments seem to rule out this latter possibility, and this work therefore raises the interesting possibility that *Acanthamoeba* myosin may in fact be a much smaller molecule than the more usual myosins.

Discussion

The movement of muscle is undoubtedly a consequence of the interaction of actin with myosin. The fact that amoeba actin so closely resembles muscle actin, despite the eons of evolution which must separate amoebas from vertebrates, makes it seem nearly certain that the amoeba actin must also participate directly in cell movements. However, certain problems must be resolved before the role of actin in *Acanthamoeba* can be specified more precisely. The organization of the cytoplasmic actin filaments during various sorts of movements must be described. The cellular localization of amoeba myosin and the mode of its interaction with actin must be defined. The role of cytoplasmic microtubules and of cytoplasmic dynein (if there is such a molecule in this or any other species) in *Acanthamoeba* must be clarified. It is clear therefore that many problems remain to be solved before motion in *Acanthamoeba* will be well understood.

Summary

Actin isolated from the soil amoeba, *Acanthamoeba castellanii* resembles muscle actin as follows: it is a globular monomer at low ionic strength and a double helical filament in 0.1 M KCl; the filaments form arrowhead shaped complexes with rabbit muscle heavy meromyosin which cannot be distinguished from similar complexes with rabbit actin; the filaments activate Mg-ATPase of rabbit muscle heavy meromyosin, and this activation can be inhibited by native tropomyosin from muscle; the amino acid compositions of amoeba actin and muscle actin, including the presence of *3*-methylhistidine, are very similar; the amino acid composition around the *3*-methylhistidine residue is very similar in amoeba and muscle actin. The role of actin in movement of *Acanthamoeba* is briefly discussed.

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Note added in proof: Direct comparison of the molecular weights of *Acanthamoeba* actin and muscle actin by electrophoresis in the presence of sodium and muscle actin by electrophoresis in the presence of sodium dodecyl sulfate shows that the proteins migrate indentically, and the molecular weight of amoeba actin is therefore 46 000 instead of 39 500 as stated in the text. (See Weihing and Korn 1972).

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Biophysical aspects of pseudopod extension and retraction in the giant amoeba, *Chaos carolinensis*

It has been said that theories of amoeboid movement tend to rush headlong in advance of hard experimental data on which proper theories should be based. This tendency is beginning to be reversed by recent experimental data which now offer a basis for a decision among several models to explain the basic functional components of amoeboid movement: pseudopod extension and retraction and the cytoplasmic streaming which accompanies these processes.

The majority of students of cell movement have long "known", at first somewhat intuitively but later with evidence of increasing rigor, that contractility must be the basis of amoeboid movement. Dr. Pollard and Dr. Weihing will discuss at this symposium the properties of isolated contractile proteins bearing some similarity to those found in muscle (Pollard and Korn 1971). This represents the first taste of fulfillment for the expectations of several generations of workers interested in amoeboid movement. We can now look forward to a rapid growth in our knowledge of the molecular constituents of the machinery of contractility, not only of muscle and amoebae, but of many other "primitive" or "non-muscular" motile systems.

Acceptance of the fact that contractility is the basis of amoeboid movement raises the question of where contraction occurs in the cell and how it is controlled during the normal behavioral pattern of the cell. The three possibilities were considered over a decade ago (Allen 1961):

(1) Ectoplasmic tube contraction might establish a positive pressure gradient along which cytoplasm streams toward "weak spots" in the plasmagel or ectoplasmic layer (Mast 1926, Goldacre and Lorch 1950, Jahn 1964).

(2) Frontal contraction might occur in the portion of the endoplasm becoming everted at each pseudopod tip, causing tension to act on the endoplasm, compression on the advancing rim of the ectoplasmic tube (Allen 1961 a).

(3) Active shearing of some layer or layers of the endoplasm might drive that material forward, reacting against the relatively stationary ectoplasmic tube (Allen 1961 a, Subirana 1970).

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Active shearing was suggested as an unlikely possibility by Allen (1961 a). More recently, Subirana (1970) has predicted quantitatively from the same model the kind of velocity profiles expected in the endoplasmic stream. Since they are opposite to profiles actually found (Allen and Roslansky 1959), there is no compelling reason to keep this model under consideration, even though it does appear to describe the events in certain plant cytoplasmic streaming systems, such as *Nitella* (Kamiya 1959, Kamitsubo 1966).

From our laboratory have come three sets of new experimental data that enable us to discriminate between the other two contractility models for amoeboid movement. I shall only summarize the three results here and discuss briefly their combined significance. The details will have been published by the time the present article appears in print.

Experimental reversal of the hypothetical positive pressure gradient suggested as the cause of streaming and pseudopod extension

The ectoplasmic tube contraction model, which attributes cytoplasmic streaming and pseudopod extension to the development of a positive internal pressure gradient, can be tested experimentally. Streaming into extending pseudopods should cease instantly if the assumed internal positive pressure gradient is abolished or reversed by the application of suction to a pseudopod.

Kamiya (1964) succeeded in demonstrating that very small pressure differences (less than 1 cm of water) could cause streaming to stop or reverse in amoebae placed in an "amoeba double chamber". At that time it was believed that the motive force was being measured, as is the case in the classical slime mold double chamber method (Kamiya 1940, 1959). In the amoeba double chamber, however, pseudopods in both chambers seemed unaffected by the applied pressure gradient.

Allen, Francis and Zeh (1972) have now performed the experiment which asks whether streaming can be reversed by an artificially induced pressure gradient. In these experiments a capillary is mode to fit tightly over a short pseudopod and measured suction is applied in such a manner that cytoplasm is either drawn into an artificial pseudopod or sucked out of the cell through the ruptured plasmalemma. In either case, the results are the same and decisive. Suction at pressure differences up to 35 cm of water caused local flow of endoplasm into the capillary but did not reverse streaming into advancing pseudopods, which continued to extend against the direction of suction almost up to the moment that the last portion of the amoeba's cytoplasm disappeared down the capillary orifice.

In interpreting this result it is necessary to bear in mind that the endoplasm is a complex viscoelastic fluid (see Section 3) and the dimensions of the cell are such that

viscous forces predominate over inertial forces: that is, the Reynolds number is very small. It is now apparent that the simple hypothesis that flow results from a positive pressure gradient present in the cell can be discarded as invalid. Models based on pressure gradients developed as the result of active shearing or other forces acting along the inner wall of the ectoplasmic tube can also be excluded.

Viscoelastic properties of the endoplasm

If, as has long been suspected (Swann 1951), a pressure gradient is not the motive force, then the question arises as to the real nature of the motive force and its localization in the cell. According to the frontal contraction model (Allen 1961 a), the endoplasm contracts as it approaches each pseudopod tip. According to this model the motive force consists of a contractile force delivered at pseudopod tips as tension on the endoplasmic stream and compression on the advancing rim of the ectoplasmic tube. Tension applied to the endoplasm must be transmitted by cross-linked structural elements in the axial endoplasm.

Evidence for the presence of structure in the axial endoplasm had arleady been obtained both from velocity profiles of "plug" flow in the endoplasm (Allen and Roslansky 1959) and from the anomalous behavior of cytoplasmic particles caused to sediment by centrifugation (Harvey and Marsland 1932, Allen 1960).

Francis and Allen (1972) have now recorded birefringence changes in the endoplasm of amoebae when suction was applied to cause artificial outward flow. A square wave of applied suction caused an immediate increase in endoplasmic flow and an approximate doubling in birefringence. The increase in birefringence did not follow the pressure step function, but increased almost linearly as a ramp wave. When the suction was suddenly released, the cytoplasm recoiled back into the cell and the birefringence dropped, again almost linearly, but not all of the induced birefringence was lost.

The interpretation of this result is straightforward. The birefringence is due not to orientation by the velocity gradient (which is proportional to the pressure difference caused by suction) but due to strain, which depends in turn on the extent of endoplasmic extension during induced flow.

Sometimes streaming stopped even while the suction was still being applied. In one case the birefringence was observed to decline slowly without visible movement of particles.

These results show that the endoplasm of *Chaos carolinensis* is viscoelastic, and thus capable of storing and transmitting forces developed at pseudopod tips. This finding is a sine qua non for the frontal contraction model, for tension cannot exist in a purely viscous fluid.

The pattern of birefringence in moving amoebae

Ever since weak birefringence was first detected in the cytoplasm of *Chaos* about a decade ago (Allen 1961 b), it has been assumed that linear elements of some kind would be found in the electron microscope as soon as fixation techniques improved to the point where these structures were adequately preserved. Nachmias (1964, 1968), Schäfer-Danneel (1967), Wohlfarth-Bottermann (1964), Morgan, Fyfe and Wolpert (1967), and Pollard and Ito (1970) have provided increasingly refined pictures of the cytoplasmic ultrastructure of amoebae. It is now possible to recognize at least two kinds of filaments, thick and thin ones. The thick ones are of as yet unknown composition, but the thin filaments (microfilaments) have been identified as amoeba actin because of their ability to bind heavy meromyosin (Pollard and Korn 1971).

While studies on fixed amoebae have been useful in the identification of at least some of the linear elements that comprise the contractile machinery of the cell, there is no doubt that amoebae are not well preserved, even at the light microscopic level by conventional fixatives. Contraction, syneresis, dislocation of materials and loss of characteristic birefringence accompany fixation of large carnivorous amoebae by conventional methods (L. Comly, unpublished).

At present, the pattern of birefringence of amoebae constitutes the only reliable information available on the relative number and degree of orientation of microfilaments and other linear elements in the amoeba.

The birefringence in amoebae is weak and very difficult to detect even with a high extinction polarizing microscope because of the presence of background light scatter from inclusions. The present studies were carried out on amoebae subjected first to centrifugation to sediment the heaviest inclusions and then immediately to microsurgery to excise portions of the cell containing high concentrations of these particles. The most nearly transparent amoebae that recover from this operation not only stream more actively than controls, but reproduce normally (Allen and Francis 1965).

Our most striking finding is that the endoplasm is always positively birefringent (Allen 1972). In many specimens we were able to record this birefringence throughout the endoplasm, from the tips of pseudopods to the recruitment zone, where the endoplasm appears to "peel off" the walls of the posterior portion of the ectoplasmic tube. This result probably indicates that the linear elements (microfilaments, etc.) in the endoplasm are at least partially oriented parallel to the stream as they enter the "fountain zone" region where contraction occurs, according to the frontal contraction model.

The ectoplasmic tube has been found to have a weaker birefringence with some patches of negative axial birefringence, especially in the region adjacent to the streaming endoplasm. This pattern is what would be expected if the ectoplasmic tube, even though constructed of positively birefringent endoplasm, was subjected to

compressive forces acting on its advancing rim. The greater reduction in birefringence in the inner region of the ectoplasmic tube is probably a result of the pattern of streaming at the tip: the peripheral endoplasm follows a shorter path into the inner region of the ectoplasmic tube and would be expected to suffer more compression than the axial endoplasm, which moves to the periphery of the ectoplasmic tube via the pseudopod tip.

The patchy distribution of negative axial birefringence corresponds to that observed by Allen, Francis and Nakajima (1965), who observed repeating cycles of photoelastic effects: positive endoplasmic and negative ectoplasmic birefringence. This was interpreted as a photoelastic manifestation of tension and compression in the regions where these forces were predicted by the frontal contraction model.

The conclusions to which these new results lead are as follows:

(1) The suction experiments summarized in section 1 clearly show no evidence that the positive pressure gradient assumed by the ectoplasmic tube contraction model is present. Instead, each pseudopod appears to be able to extend against a formidable pressure gradient. Hence, the model is untenable.

(2) The same suction data as well as earlier measurements of endoplasmic velocity profiles similarly invalidate the active shearing model.

(3) The frontal contraction model now emerges as the only explanation of amoeboid movement that is consistent with all of the experimental results. The demonstration of endoplasmic viscoelasticity (section 2) confirms one of the underlying assumptions of the frontal contraction model; namely, that the endoplasm must have structure capable of transmitting tensile and compressive forces.

(4) The pattern of birefringence in living amoebae (section 3) is the most reliable indication of the local concentration and orientation of linear elements in these cells. To at least some extent, the degree of orientation observed appears to be the result of the motive force acting on viscoelastic elements in the cytoplasm. The pattern observed is quite consistent with the predictions of the frontal contraction model.

Acknowledgements

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Summary

The results of the new experimental approaches designed to explore the biophysical aspects of pseudopod extension and retraction in *Chaos carolinensis* are reported and discussed in relation to plausible contractility models for amoeboid movement. Suction experiments

designed to test the classical positive pressure gradient theory showed that streaming into pseudopods proceeded unaffected by the establishment of a strong negative pressure gradient. This result excludes any model which explains cytoplasmic streaming as pressure induced flow. Measurements of endoplasmic birefringence changes caused by suction demonstrated that amoeba endoplasm has viscoelastic properties, as would be expected from the presence of cross-linked microfilaments and other linear elements. The normal pattern of birefringence has been recorded photographically. Pseudopodia show, as a whole, weakly positive axial birefringence. The strongest positive birefringence is found throughout the endoplasmic stream. Weaker positive birefringence is found in the outer region of the ectoplasmic tube. The inner region of the ectoplasmic tube often shows patches of weak negative birefringence. The results are uniquely compatible with the frontal contraction theory of amoeboid movement.

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Membrane-turnover during locomotion of *Amoeba proteus*

The cell membrane of protozoa and especially of amoebae is exposed to particular stresses: in addition to a regulatory function concerning the exchange of substances, the cell membrane is responsible for the perception of both chemical and physical excitations as well as the production of adhesive forces causing the contact between cell body and substratum. Furthermore, if pseudopod extension and retraction can be regarded as a general feature of amoeboid movement, then it is possible to consider the role of the cell membrane from two points of view: (1) its mechanical role in movement and (2) its role in controlling cytoplasmic streaming, particularly in relation to electrical phenomena. Since Wolpert and Gingell (1968) recently reviewed the electrophysiological properties of the membrane of *A. proteus* by setting up a new theory, this paper will be restricted to summarizing the present state of knowledge concerning the mechanical properties of the plasmalemma during cellular locomation. In this connection it is of interest to note that the cell membrane can now be excluded as an agent in providing the motive force. The cell membrane is involved in amoeboid movement in a more passive sense and there are four main possibilities as to how the membrane may behave when the cell moves and changes its shape (Fig. 1).

The classical concept of membrane behaviour in amoebae (Fig. 1 a) was put forward by Jennings (1904) and Mast (1926) and met further support from Griffin and Allen (1959). These authors suggested the cell membrane to be a rather permanent structure with a slow turnover, which is plastic and can flow in order to accommodate changes in cell shape. It should roll over the cell body as an air-balloon half filled with water rolls over the ground.

A quite contrary view of the moving behaviour of the amoeboid cell membrane has been expressed by Goldacre and Lorch (1950) and Goldacre (1961). In the opinion of these scientists (Fig. 1 b) the plasmalemma is resorbed in the uroid region and formed de novo at the front. The membrane itself is in a stationary condition and remains at rest along the sides of the cell body. In consequence of this theory the rate of membrane-turnover must be high during movement and the cell

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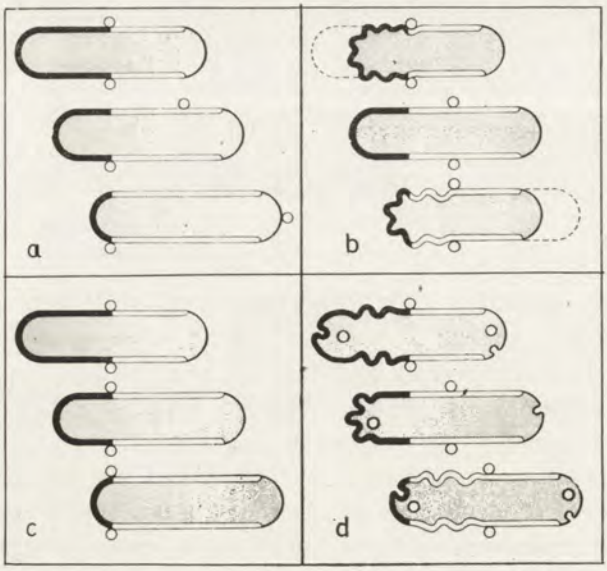

Fig. 1. Schematic representation of different possibilities as to how the cell membrane of *A. proteus* may move and how membrane–turnover may occur (for description see text; after Komnick et al., in press)

may be expected to renew its membrane completely each time it passes through its own length, that is every 4–5 min.

In interpreting the mechanical properties of the cell membrane of *A. proteus*, Czarska and Grębecki (1966) developed a conception based on folding and re-folding processes (Fig. 1 c). They believed the cell membrane to be a structure with a slow rate of renewal, which shifts forward together with the whole cell body. The expansion of surface area was explained by membrane unfolding, and the collapse by re-folding processes. It follows that shifting of cell membrane during amoeboid movement occurs discontinuously by alternating prolongation and shortening of the whole cell body.

Since arguments for and against these concepts could be put forward in the last years by discussions and different experiments but without favouring one of the three theories, extensive light- and electron microscopical investigations as well as cinematographical studies were started in order to elucidate the dynamic and mechanical properties of the cell membrane of *Amoebae* of the *proteus* type. The results of these investigations are presented in Fig. 1 d and can be shortly summarized as follows:

The cell membrane of *A. proteus* must be considered as a structure with long-time constancy in surface area. During locomotion the entire membrane slides forward along the outer ectoplasmic surface of the cell body. This process involves simultaneous folding and unfolding of the plasmalemma in neighbouring regions at the posterior end. In addition, permanent endocytosis at the uroid slowly reduces

the cell membrane area, while exocytosis presumably at the front region is the source of membrane regeneration.

In the following newer experimental results attempting to answer the question of the nature and rate of membrane-turnover in *A. proteus* will be reported.

Results and discussion

Mechanism and rate of membrane-turnover

A. proteus is a unicellular organism which constantly changes its shape and moves by cytoplasmic streaming (Pl. I 1). The motile form shows either polypodial or monopodial (Pl. I 1 a) differentiation and is anterior-posterior orientated. The posterior part, also called uroid, or tail region is characterized as an area with high folding degree and considerable outline surplus (Pl. I 1 b), whereas the anterior part of monopodial cells is smooth-outlined. In studying the nature of membrane resorption during normal locomotion of both monopodial and polypodial cells it could be demonstrated by light- and electron microscopical investigations (Stockem 1966, Wohlfarth-Bottermann und Stockem 1966, Stockem and Wohlfarth-Bottermann 1969) that membrane loss occurs as the result of a slow permanent endocytosis at the high folded uroid region. The separate stages of this permanent endocytotic process may be summarized as follows (Pl. II 2).

The membrane of small hyaline pseudopodia continuously formed during locomotion at the tail of *A. proteus* invaginates at its tip and a broad channel arises leading into the cell interior (Pl. II 2 a). This is followed by extension and enlargement of the endocytosis channel as the result of rapid elongation of the pseudopodium (Pl. II 2 b). Then a vacuolar dilatation of the channel base (Pl. II 2 c) pinches off and is carried by the cytoplasmic stream to the interior of the cell (Pl. I 2 d). Finally, the remainder of the channel disappears. The average width of the channels is between 1 and 2 mμ and their average length 10 to 20 mμ. They seldom last longer than one minute and generally one to three channels are simultaneously formed during active amoeboid movement.

Endocytotic activity (Pl. III 3) can also be demonstrated by electron microscopical investigations using Aerosil (Silicium dioxide) as a tracer substance. Aerosil is a labelling substance which does not affect normal endocytotic cell activity (Pl. III 3 a) and which behaves as an inert material with no other action than to enable the content of endocytotic vacuoles to be identified with certainty (Stockem 1967). The marker substance is adsorbed onto the mucous layer of the cell surface, then ingested by the channel-like membrane invaginations and, finally, transported into the cytoplasm by pinched off endocytotic vacuoles (Pl. III 3 b), which are called endosomes (Stockem und Wohlfarth-Bottermann 1969).

Because conclusive quantitative studies concerning the amount of cell surface

ingested by permanent endocytosis were still lacking (Wolpert and Gingell 1968), measurement of membrane uptake in the uroid region was attempted by combined electron microscopical and morphometrical methods as introduced by Weibel et al. (1966). Those investigations were realized by the production of large supporting films (Stockem 1970) covering preparation grids with holes of 1 or 2 mm in diameter. Ultrathin sections through complete mono- or polypodial amoebae (Pl. IV 4) treated for different periods with an Aerosil solution could thus be mounted on such grids and observed in the electron microscope. Each amoeba was photographed then at a primary magnification of 2500× by taking more than 50 single, overlapping negatives. After enlarging these negatives 3×, a montage could be assembled exhibiting the whole amoeba at a final magnification of 7500×. This magnification was suitable for the identification of all endosomes which contained marker substance. The presented *A. proteus* (Pl. IV 4 a) was treated with Aerosil for 45 min and photographed at a lower magnification of 250× in order to give a representative survey. A reduced drawing from the original plane (Pl. IV 4 b) indicates the amount, size and distribution of the formed endosomes, which are pointed out by black colour in this case. Subsequently the marked endosomes could be plotted morphometrically by a screen of special lines (Fig. 2). This type of screen was developed

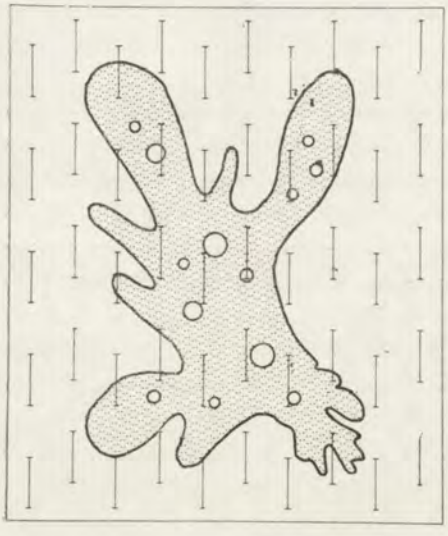

Fig. 2. Schematic representation illustrating the method used to determine the rate of membrane turnover during normal locomotion of *A. proteus*. The screen of special lines was developed by Weibel (1966); (for description see text)

by Weibel et al. (1966) and is characterized by displaced short lines arranged in rows of respectively the same distance. After covering the photographic montage by such a screen the cutting-points of either endosomal membranes or the plasmalemma with the screen-lines could be counted. Setting the cutting-points of more than 20 amoebae into a percentage relation the following average values were obtained (Table 1), monopodial cells treated with Aerosil for 30 min ingested 4.2% of their

Table 1

Membrane turnover in *A. proteus* measured with Aerosil as a tracer substance

Endocytosis	Type of locomotion	Incubation-time in min	Membrane-uptake in % cell-membrane-endosomes	Membrane turnover in % per min
Permanent	monopodial	30	4,2	0.14
	polypodial	45	6.4	0.14

total cell area by permanent endocytosis, whereas polypodial cells during the time of 45 min active locomotion in the marker substance resorbed 6.4% of their surface area. Converting these values to a surface uptake per minute reveals equal endocytotic activities of 0.14% in both mono- and polypodial cells and demonstrates good experimental agreement. From the morphometrical studies it may be concluded that there is no evidence for rapid resorption or restoration of cell surface during normal locomotion of *A. proteus* as postulated by Goldacre and Lorch (1950). The surface area of this organism is only slowly reduced by permanent endocytosis at the uroid region. The measured value of 0.14% membrane turnover per minute corresponds to a time of 12 h for replacing an area of surface equal to the total area of the cell. This is nearly consistent with the data published by Wolpert et al. (1964), and Chapman-Andresen (1963). These authors suggested a membrane turnover of 0.2% per minute. In addition it is of interest that Hausmann (1971) has just finished investigations concerning the membrane turnover in the small Limax-amoeba *Hyalodiscus simplex*. The values obtained in this study indicate membrane resorption by permanent endocytosis at the tail region of about 0.2% per minute. This means slow membrane turnover in this species, too, and supports our view and the opinion of other authors (Wolpert and Gingell 1968, Czarska and Grębecki 1966) that the membrane resorption-regeneration cycle plays only a rather secondary role in amoeboid movement. Nevertheless, it is our belief that permanent endocytosis is very important for the continuous uptake of smaller food particles, as can be demonstrated in feeding experiments with bacteria and model substances of similar size.

Intracellular fate of membrane

In the following, experiments (Hausmann 1971) elucidating the intracellular fate of the ingested cell membrane as well as studies concerning the source of membrane regeneration will be briefly dealt with.

The endosomes, arising as a result of permanent or induced endocytosis, undergo an intracellular fate reminiscent of the lysosomal concept of intracellular digestion proposed by de Duve (1963) and others. Using Thorotrast as a marker substance it was possible to prove an intracellular digestion cycle in the Limax-amoeba *Hyalo-*

discus simplex, which is similar to the one suggested for *proteus*-amoebae (Holter 1965, Stockem and Wohlfarth-Bottermann 1969) and may represent intracellular digestion in amoebae in a more general sense (Fig. 3).

After adsorption of the marker substance onto the mucous layer and formation of an endocytosis channel, the base of the channel dilatates (No. 1) and so-called primary endosomes (No. 2) are pinched off. The primary endosomes, hitherto very irregular in shape, show a varying subsequent fate: some of them coalesce by an intracytotic process with older endosomes (No. 5), which generally contain food

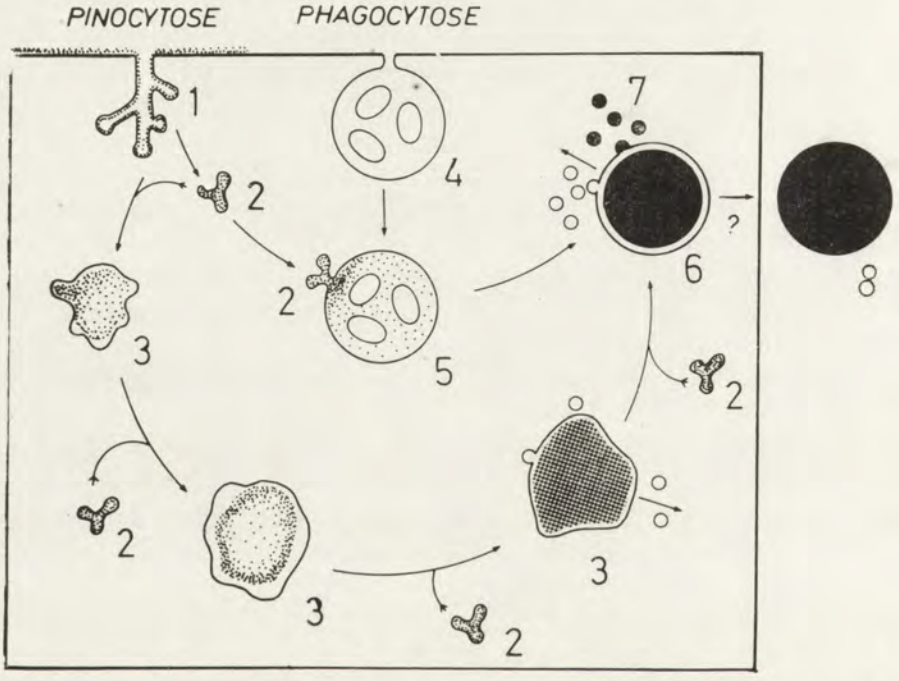

Fig. 3. Schematic representation of the intracellular digestion cycle in the Limax-amoeba *Hyalodiscus simplex* using Thorotrast as a marker substance (for description see text; after Hausmann 1971)

particles such as bacteria in advanced states of digestion. As these food vacuoles had been formed by phagocytosis previous to addition of Thorotrast to the culture medium (No. 4), they can be regarded as preexistent lysosomes (Thoenes et al. 1970). Fifteen minutes after beginning the experiment their content is already marked by Thorotrast (No. 5). Other primary endosomes fuse together thus forming larger secondary endosomes (No. 3). The next event following formation of secondary endosomes is condensation of the endosome content which advances until electron-dense aggregates have arisen (No. 6). The same changes occur with the content of the preexistent lysosomes, and about 6 h after the addition of Thorotrast to the

culture medium secondary endosomes as well as the fusion-products of primary endosomes and preexistent lysosomes are transformed into lysosomal vacuoles (No. 6). Subsequently small vesicles (No. 7) are pinched off from these lysosomal vacuoles by a process of intracytosis, or – as it has been called by other authors (Holter 1965) – micropinocytosis. The significance of these vesicles, which can also be demonstrated in other protozoa (Favard and Carasso 1963, 1964), remains unexplained. It is presumed that they contain digested substrate. Further experiments should prove their conclusive fate as well as the origin of different lysosomal enzymes needed in the process of digestion. These enzymes have been recently demonstrated in *A. proteus* by Chapman-Andresen and Lagunoff (1966) on the light microscopical level and by Wise and Flickinger (1970 b) on the electron microscopical level.

Defecation and mechanism of membrane regeneration

The defecation mechanism of the digested or condensed lysosomal content remains to be elucidated. The lysosomal content may either be re-excreted from the cell by exocytosis or – similar to milk-secretion – extruded by a pinching off process. In the first case the lysosomal membrane should coalesce with the cell membrane, enlarging the surface area in this way, whereas the pinching off process must be followed by reduction of cell surface area. Investigations in *Hyalodiscus simplex* (Hausmann 1971) seem to prove a third possibility (Pl. V 5).

The content of the lysosomes consisting of membranous structures and condensed Thorotrast (Pl. V 5 a) can be found as balls attached onto the mucous layer of the cell surface (Pl. V 5 b) 20 h after application of the tracer substance to the amoebae. These defecation balls, which are carried about by the cells for several days, are secondarily infected with living bacteria and lack a surrounding membrane as can be clearly seen in Pl. V 5 b. Hence, it follows that a pinching off process as previously described can be excluded. Normal exocytosis may also be excluded by virtue of the lack of an intact mucous layer on the inner surface of the lysosomal membrane as well as by our failure to demonstrate naked surface areas without a well developed glycocalyx.

The expected defecation mechanism explaining these contradictions is represented in Fig. 4. The defacation may occur in three steps:

After fusion of the lysosomal membrane with the plasmalemma a connection between the cell interior and the exterior is produced by which the defecation ball can leave the cytoplasm. The membrane of the lysosomal vacuole, which, in contrast to the plasmalemma is not covered by a mucous layer, is then pinched off again and the reformed empty vacuole passes back into the cytoplasm. This mechanism is strongly supported by studies of Schneider (in press) on the contractile vacuole and should be further supported by subsequent experiments. As morphological evidence that the defecation process contributes to any great extent in membrane restoration was lacking, another regeneration mechanism was sought, which would

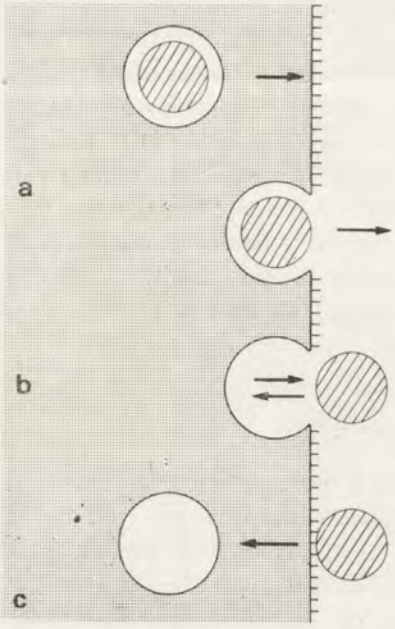

Fig. 4. Schematic representation of the mechanism explaining the defecation of the undigestible content of lysosomes; a — fusion of the lysosomal membrane with the plasmalemma, b — defecation of the lysosomal content, c — pinching off of the empty lysosomal vacuole

compensate membrane reduction occurring continuously by permanent endocytosis. In this connection it is of interest that success has recently been reported (Stockem 1969) in demonstrating a type of vacuoles (Pl. VI 6) which contains a completely differentiated mucous layer. As could be demonstrated by labelling the exterior medium with marker substances these vacuoles do not arise from the cell membrane and therefore are not identical with endosomes. Cytochemical investigations reveal that these vacuoles are formed by the dictyosomes of the Golgi apparatus and that they are responsible for the regeneration of the cell membrane. In consequence, they were called exocytotic vacuoles (Stockem 1969). After fixation in Os/Cr and subsequent treatment with $FeCl_3$ for some hours, the ends of the cisternae on the dictyosome concave face, which are dilated and contain filamentous material, are stained clearly (Pl. VII 7). On the other hand, there is only little or no staining of the cisternae on the convex face. Small vesicles adjacent to the concave face of the dictyosomes are also stained. They appear to develop into the afore mentioned exocytotic vacuoles. As our results are consistent with investigations of Wise and Flickinger 1970 a, and Nachmias (1966) it may be concluded that the Golgi apparatus of *proteus*-amoebae has a morphogenetic as well as secretory function. It seems not only to be responsible for the continuous forming of new plasmalemma, but is also engaged in synthesis of the mucous layer of the cell membrane. In consequence, it is comparable in function with the Golgi apparatus of many metazoen cells, which is now regarded as a membrane depot as well as a generation centre of mucous substances (Favard 1969).

Summary

The present state of knowledge concerning membrane-turnover in *proteus*-amoebae may be summarized as follows (Fig. 5).

The cell membrane of *A. proteus* is characterized as a structure with a rather constant surface area. During amoeboid movement the membrane is continuously reduced by permanent endocytosis at the uroid region and simultaneously regenerated by exocytosis probably at the tip of advancing pseudopodia. The endosomes, arising as a result of permanent endocytosis, undergo intracellular digestion similar to the lysosomal concept developed by de Duve (1963). It is supposed that the defecation of the lysosomal content does not contribute in any sizable

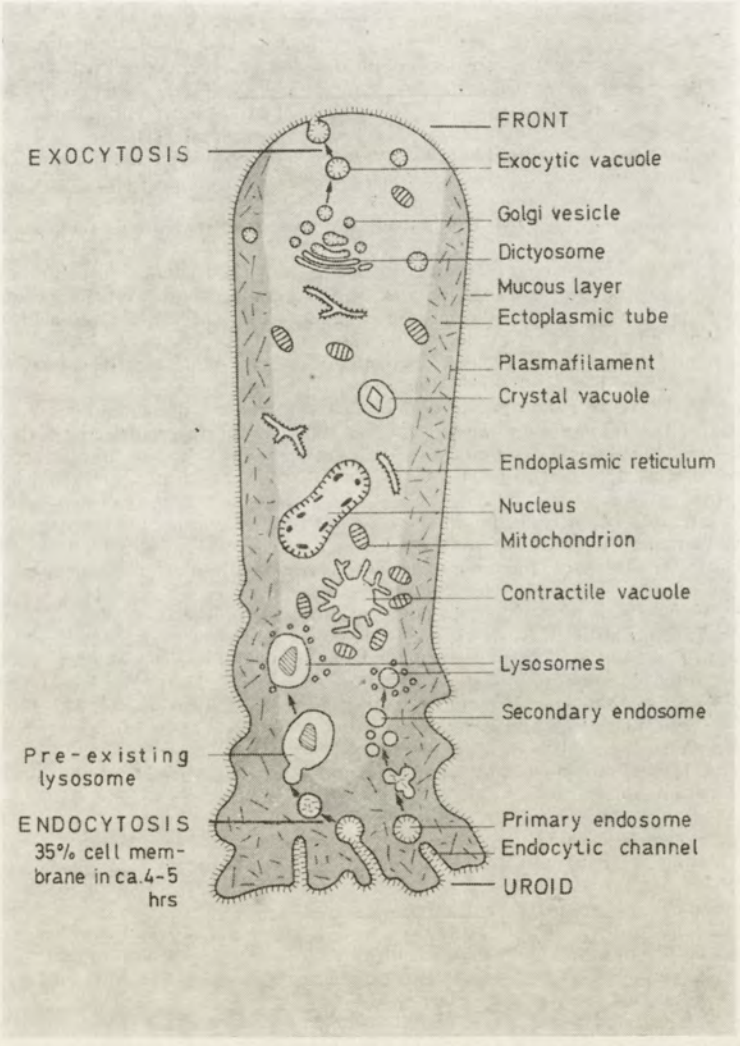

Fig. 5. Schematic representation of the different mechanisms of membrane turnover (for description see text)

degree to membrane restoration, but that the Golgi apparatus can be regarded as the regeneration source for both mucous layer and plasmalemma. The morphometrically demonstrated membrane turnover of 0.14% per minute reveals that *A. proteus* requires a time of 12 h for replacing its surface area once. Therefore, it may be concluded that the membrane resorption-regeneration cycle by means of endo- and exocytosis is of minor importance for the locomotion of these amoebae.

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EXPLANATIONS OF PLATES I–VII

1: a—Monopodial *A. proteus*. In contrast to the smooth-outlined anterior part (VE), the posterior end (HE) and the middle region (X) of the cell surface show a considerable outline surplus. The surface folding can be demonstrated more clearly at higher magnification (b). The arrow (a) indicates streaming direction. PV—pulsating vacuole. a—300×; b—1000× (After Stockkem 1970)

2: Single stages of permanent endocytosis at the tail region of *A. proteus*. a—membrane invagination and formation of a broad channel; b—beginning dilatation of the channel base; c—extension and enlargement of the channel by rapid elongation of the pseudopodium; d—formation of an endocytotic vacuole (endosome). EC—endosome; NP—food particle. 1100× (After Stockkem 1970)

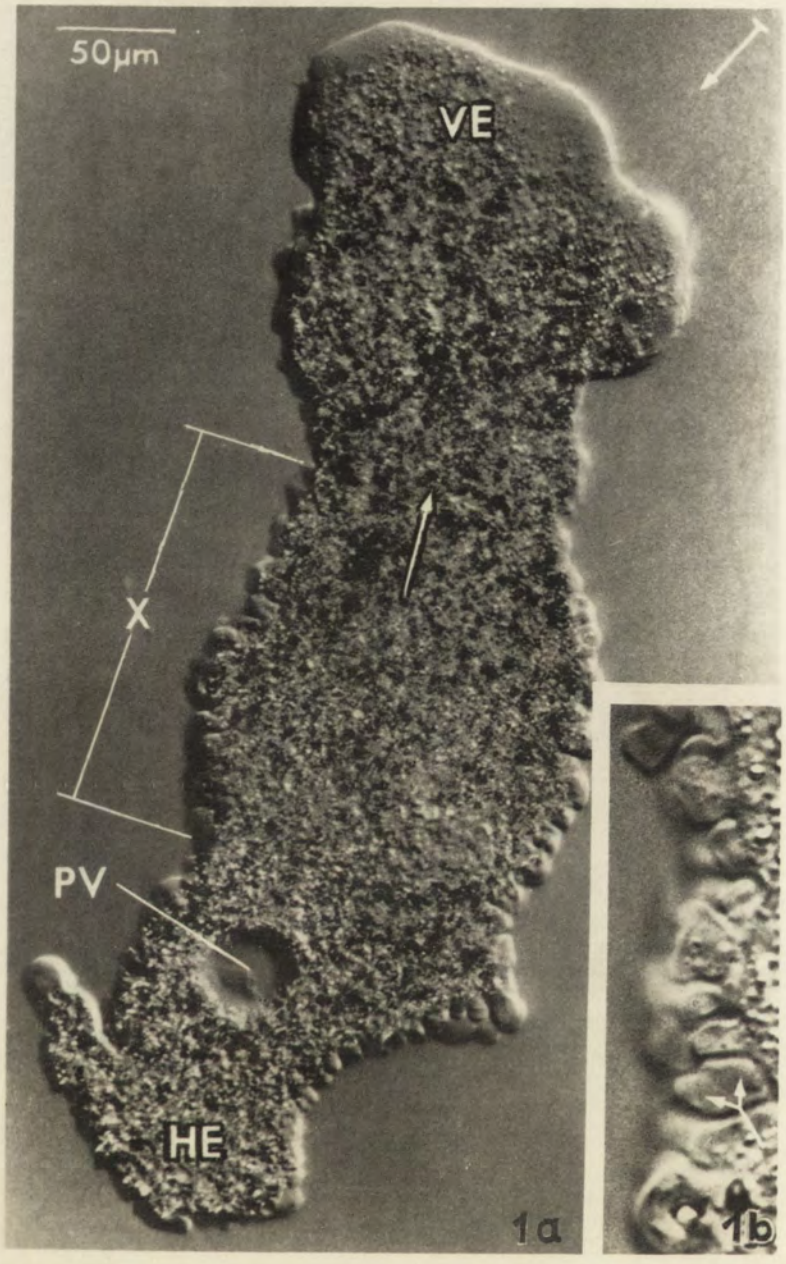
3: Sections of the tail region of *A. proteus* after 30 min of permanent endocytosis in an 0.05% Aerosil suspension at lower (a) and higher (b) magnifications. The endosomes (PV) contain large amounts of Aerosil (Ae). PK—endocytotic channels; EK—ectoplasm; EN—endoplasm; M—mitochondria; MP—micropseudopodia. a—5000×; b—10 000× (after Wohlfarth-Bottermann and Stockem 1966)

4: a—Ultrathin section through a complete monopodial *A. proteus* treated with Aerosil I for 45 min during normal locomotion. The amount, size and distribution of the formed endosomes are represented by a reduced drawing (b) from the original montage. N—nucleus; HE—posterior end; VE—anterior end; 750×

5: Ultrathin sections of *Hyalodiscus simplex* after incubation in a Thorotrast solution for 20 h; a—fusion product of primary endosomes and a preexistent lysosome. The content of the lysosomal vacuole (L) consists of membranous structures (Nr) and condensed Thorotrast (TTh), b—defecation ball (D), which is attached to the cell surface and secondarily infected with living bacteria (B). The defecation ball lacks a surrounding membrane. 14 000× (after Hausmann 1971)

6: Ultrathin sections through *Ch. chaos* after treatment with $FeCl_3$. Mucous filaments in young (Ex_1) and somewhat older (Ex_2) exocytotic vacuoles show strong reaction for $FeCl_3$ (Fe_1 and Fe_2); a—50 000×; b—70 000× (after Stockem 1969)

7: Cross-sections through single dictyosomes of *A. proteus* after treatment with $FeCl_3$. The filamentous material (Fe_1 and Fe_2) in the dilated cisternae of the dictyosome convex face (SP) is stained clearly. There is no staining of the cisternae of the concave face (RP). Filaments (FFe_3) in the Golgi-vesicles (GV) are also stained. 100 000× (after Stockem 1969)



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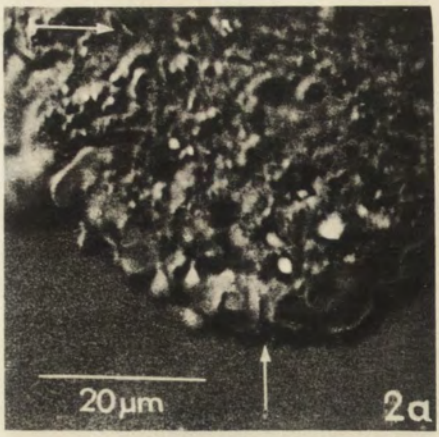

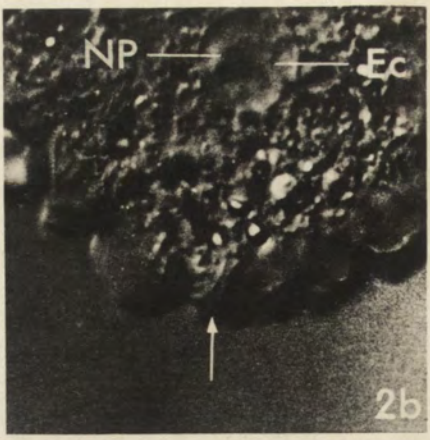

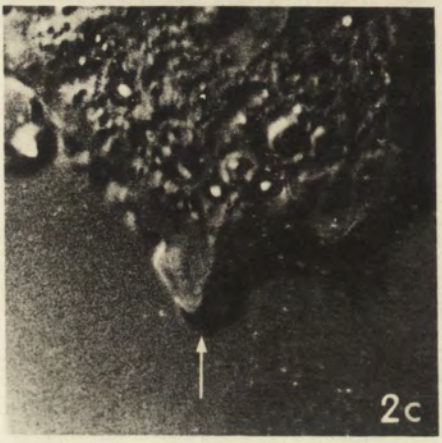

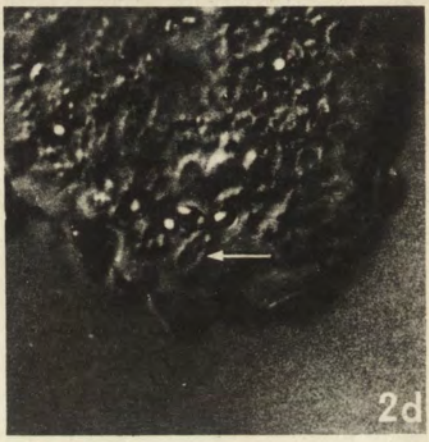

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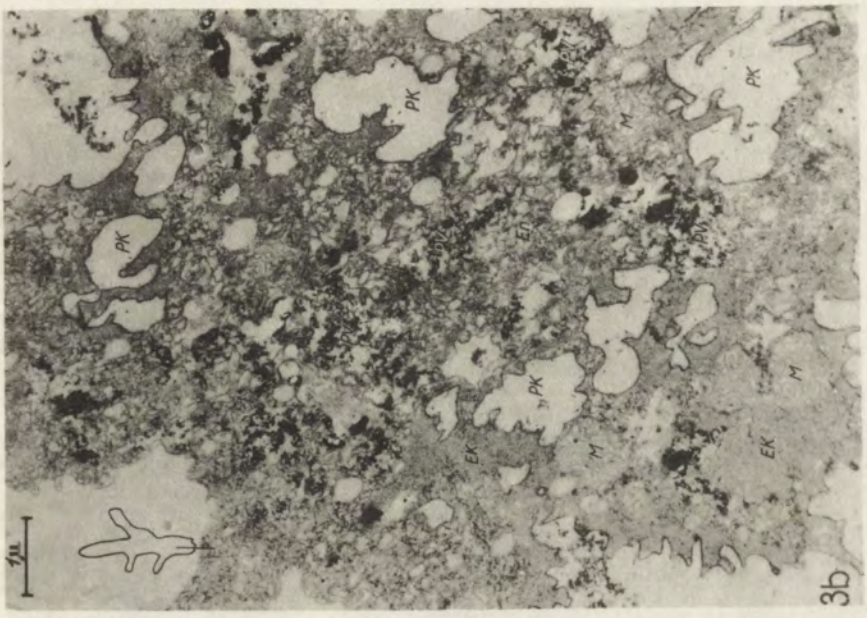

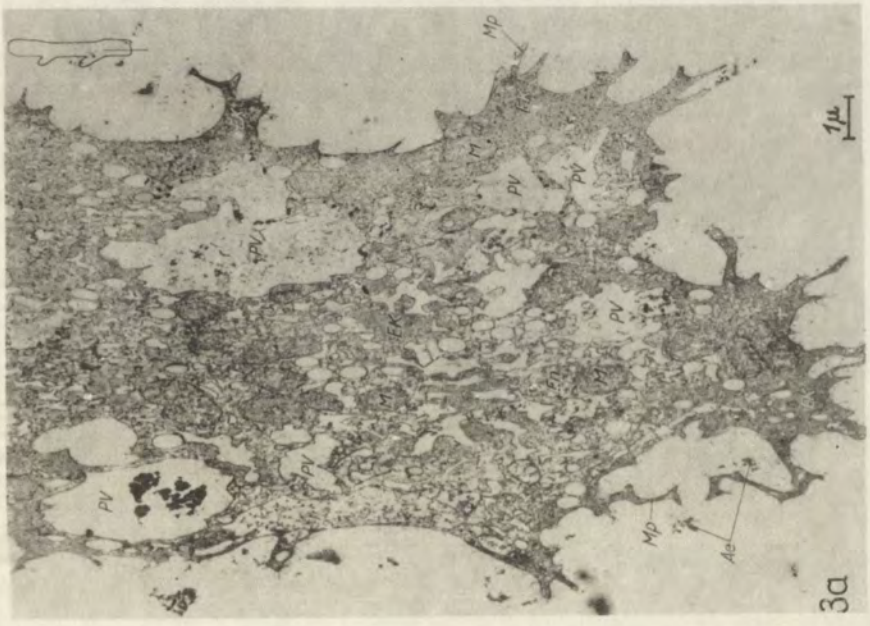

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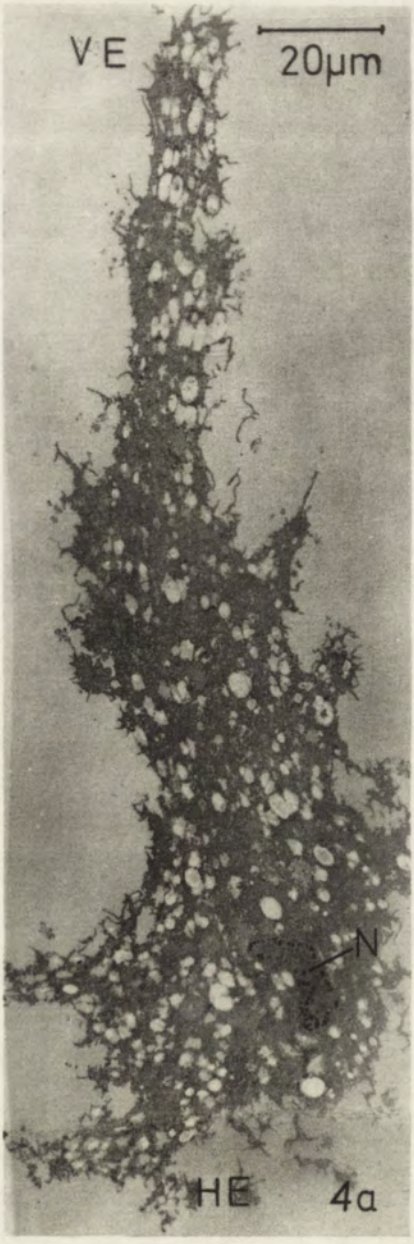

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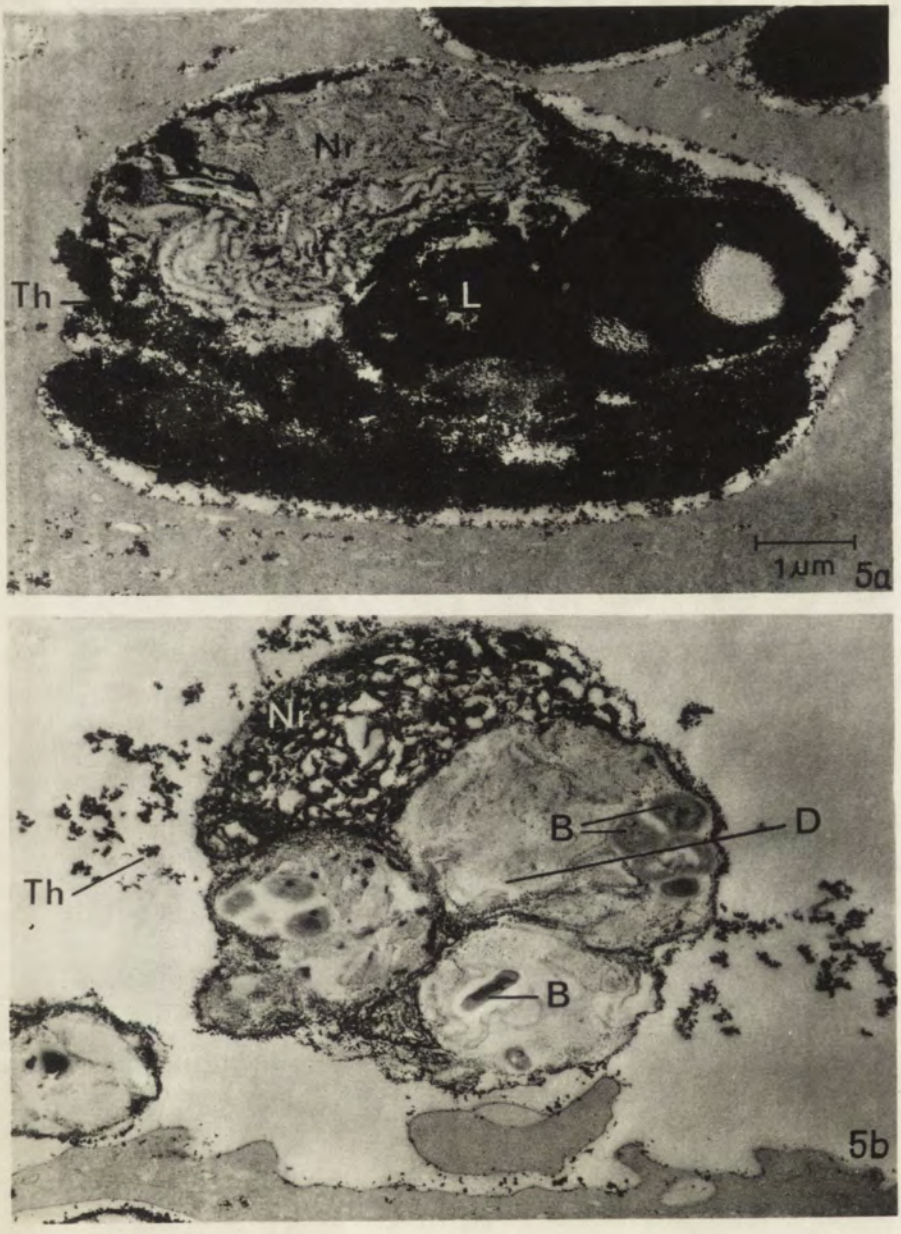

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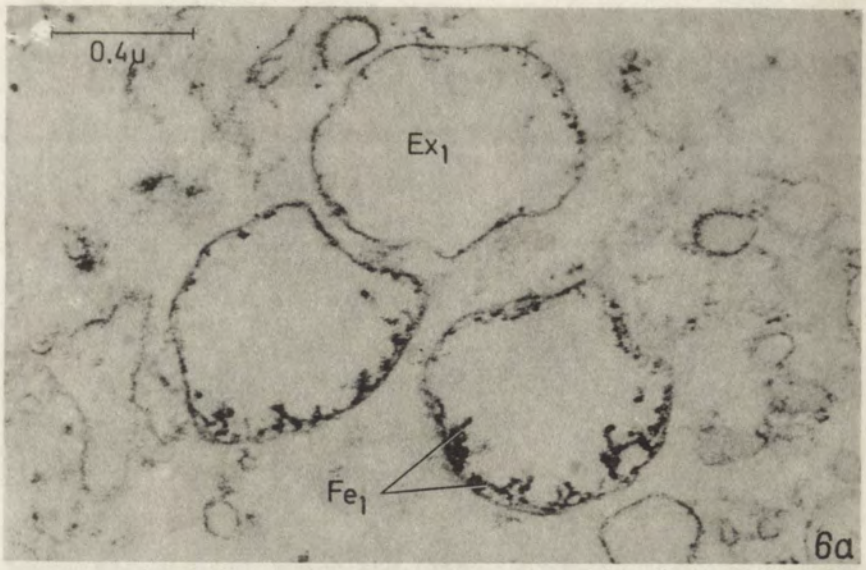

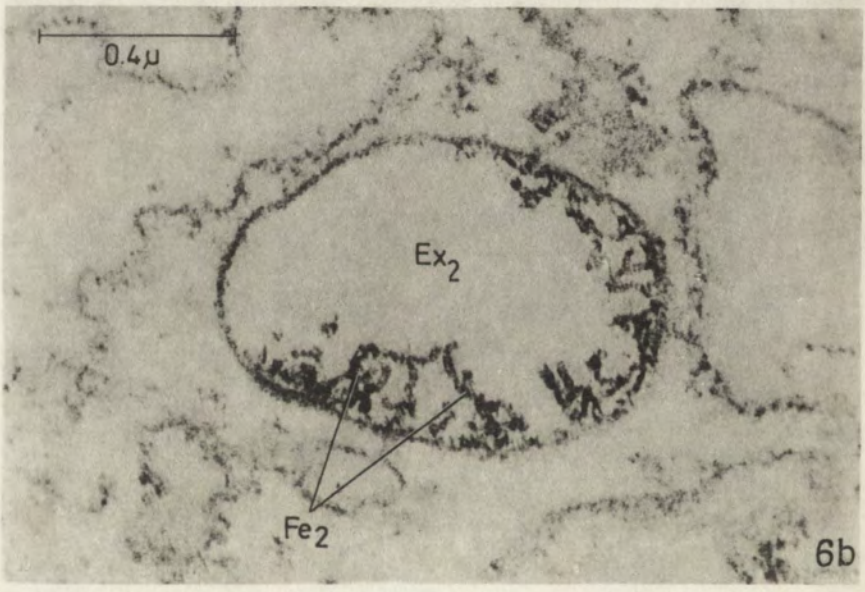

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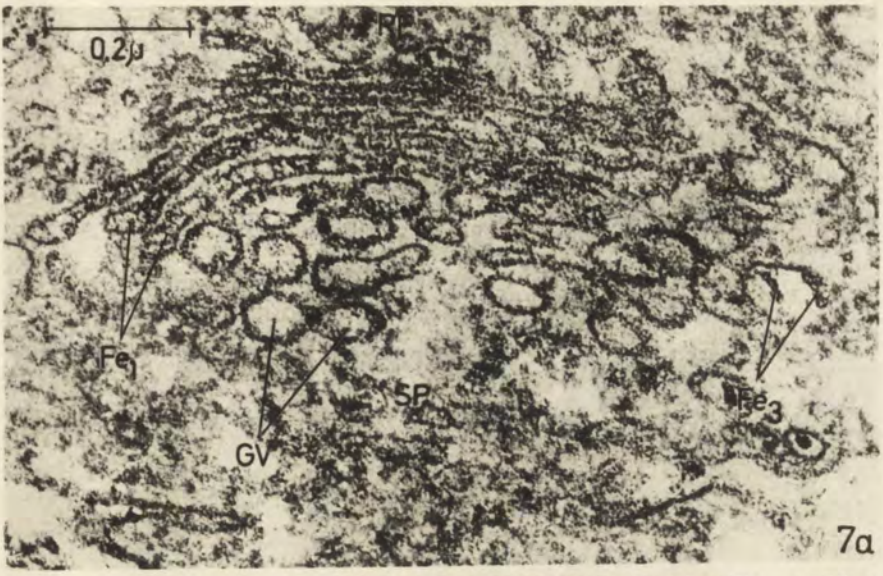

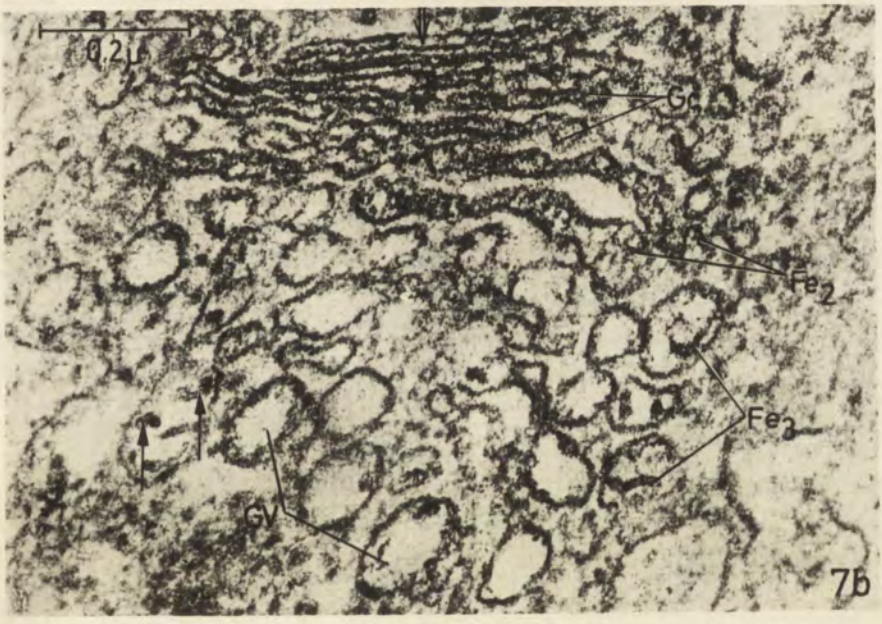

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Cinematography of cell membrane behaviour and flow phenomena in *Amoeba proteus*

Since the beginning of the 19th century when Dujardin (1835) started research work on the motility of protozoans, amoebae and amoeboid movement have been a favourite subject with a great number of workers. In recent years electron microscopy and biochemistry have opened up new lines of research, giving evidence of structure and function down to macromolecular level. In view of the large quantity of data that has been collected during more than a century, it is astonishing that attempts to combine these facts into a functional model explaining how processes on the molecular level finally enable the cell to move into a certain direction have been few and relatively unsuccessful. Unfortunately, due to the limitations in both electron microscopy and biochemistry of using only fixated or homogenized material, the results of these experimental methods represent only the static view. It therefore seems necessary to analyse the dynamic aspect of amoeboid movement as seen in the light microscope and compare the results with electron microscopical and biochemical findings in order to obtain a complete view of structures, functions and interactions taking part in the locomotion of amoeboid cells.

Material and methods

For this reason locomotion of *Amoeba proteus* cultivated in Chalkley medium was recorded on motion picture film with a Zeiss-Mikro-Kino-Kamera equipped with a microscope (Zeiss, Standard WL) and differential interference optics (Nomarski). Single frame analysis was carried out on an Lytax analyser which allowed graphic reproductions directly from the projection screen. In some cases the amoebae were marked with Latex beads (⌀ 1.009 µm) which adhere to the mucous layer of the plasmalemma and demonstrate the movement of the cell membrane during locomotion. (For further reference see: Haberey, Wohlfarth-Bottermann and Stockem (1969).

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Results

Analysis of motion picture films of *Amoeba proteus* shows two main dynamic phases of cytoplasm:

(1) The relatively rigid ectoplasmic gel tube coating the inner side of the plasmalemma and containing inclusions (vesicles) that remain stationary to the substratum (Pl. I 1 EK).

(2) The more fluid endoplasm moving inside the ectoplasmic gel tube to the anterior tip of the cell. Particles move during the exposure time and appear as streaks in Pl. I 1 (ES).

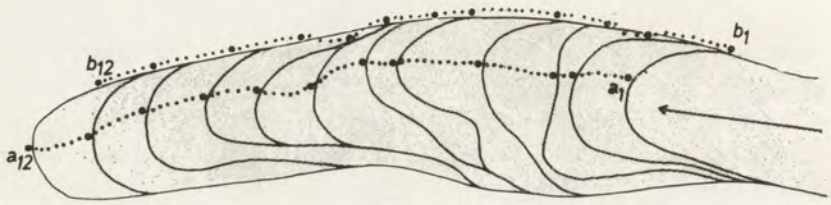

Fig. 1. Behaviour of Latex-particles adhering to the mucous layer of an advancing pseudopod. Time interval between successive stages: 6 sec

Transformation from rigid to fluid phase is mainly located in posterior regions, whereas transformation from endoplasm to ectoplasm can mostly be found in advancing pseudopods (Pl. I 2b TZ).

Transformation and flow phenomena as they appear in motion picture analysis are demonstrated in Pl. I 2a.

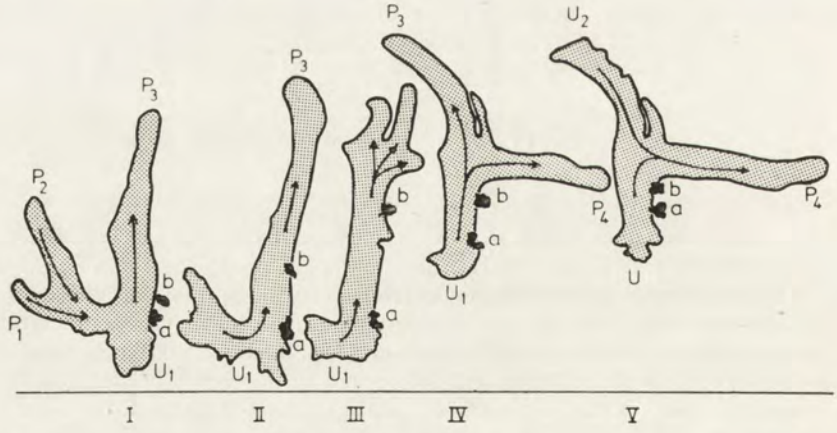

Fig. 2. Movement of marker particles at the uroid of *Amoeba proteus*. Note the alternating change of distance between the active charcoal particles a and b. P_1–P_4 = pseudopods; U_1–U_2 = retracting areas

Cytoplasmic material is carried forward by the main endoplasmic stream (E.S.) into the advancing tip. There, transformation takes place raising the viscosity of the cytoplasm. The endoplasm streams up to the anterior area of the cell membrane and is there deflected towards the periphery of the pseudopod where transformation is completed and movement stops. Transformation at the tip results in continuous deposition of plasma material at the periphery whereby the ectoplasmic gel tube and, with it, the whole pseudopod are elongated.

The newly built ectoplasm now remains stationary during the following phase of locomotion until it becomes part of the posterior region. In the uroid a contraction of the whole ectoplasmic tube is demonstrated by a shortening of the distance between neighbouring ectoplasmic inclusions. By single frame analysis of the motion picture film this contraction can be shown clearly (Pl. III 3b). Shortening and contrac-

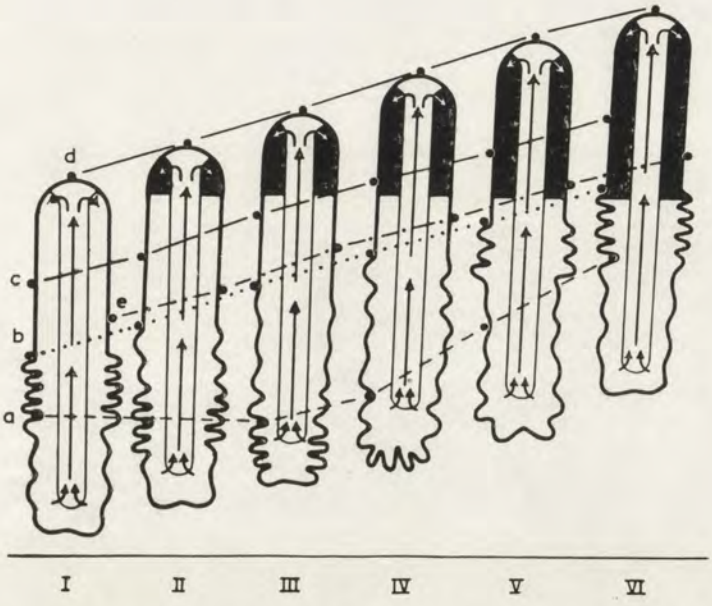

Fig. 3. Tin wire model used for simulation of marker particle behaviour, a–e = marks at the wire. For further explanation see text

tion of the uroid region leads to a volume diminution of the whole posterior cell part. During this contraction ectoplasm is again transformed into endoplasm and carried away with the endoplasmic stream to contribute to the elongation of the advancing pseudopod in the anterior region.

Transformation of ectoplasm into endoplasm in the uroid region starts at the inner periphery of the gel tube and moves slowly outward towards the plasmalemma. In this way more and more ectoplasm is transformed and carried away by the endoplasmic stream. The uroid contraction together with the deminution of volume in

the posterior region causes a shortening of the uroid during this process which leads to a surplus of plasmalemma at the cell surface. The membrane therefore appears wrinkled or folded which can readily be seen with differential-interference optics Pl. III 4 and can also be demonstrated with the electron microscope. By joining single electron micrographs a reconstructed picture of *Amoeba proteus* can be obtained and it can be used for determination of plasmalemma ratios along the outer cell surface.

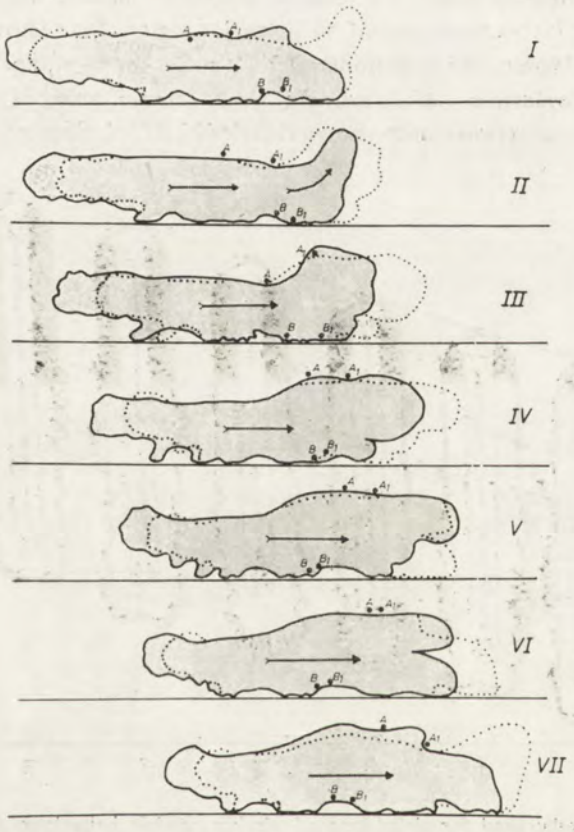

Fig. 4. Profile view analysis of *Amoeba proteus*. The marker particles (A and B) move along the periphery of the cell with approximately the same speed as the advancing tip. Successive contures are shown by dotted lines. Successive position of markers carry an index (A_1, B_1). Time interval 25 sec

By comparative measurement it can be shown that the actual surface area in the posterior part of the amoeba consists of more than 2× as much membrane as would be needed to cover the same volume by a smooth surface. In that way surplus membrane is provided and can be drawn over the cell surface to areas where plasmalemma is required for elongation or extension. The overflow of membrane

at the uroid can be understood as a reservoir of plasmalemma which enables the cell to change its shape freely.

Several theories for movement of cell membrane in *Amoeba proteus* have been proposed by a number of authors. De novo synthesis of membrane could provide the plasmalemma needed at the advancing tip to cover the volume of endoplasm streaming continuously into the pseudopod. (Goldacre and Lorch 1950, Goldacre 1961). According to Dr. Stockem's results 1971 the rate of membrane turnover by cytotic mechanisms is too low to explain an extensive synthesis of membrane as would be required for this theory. The building of new membrane and uptake of plasmalemma therefore can only be expected on a molecular basis.

As a second possibility the membrane may be visualized as possessing a rolling movement, where plasmalemma at the upper side of the cell would move with double the velocity as the cell itself. (Jennings 1904, Mast 1926, Abé 1962, Griffin and Allen 1959, Seravin 1964).

The third model proposes a gliding of the membrane along all sides of the advancing cell. Here membrane velocity should be the same as that of the cell itself. (Czarska and Grębecki 1966, Haberey, Wohlfarth-Bottermann and Stockem 1969). Actual movement of the cell membrane can be demonstrated by marker particles.

In this case the mucous layer of the cell membrane was marked with Latex beads (a and b in Fig. 1) and the trace of these beads was recorded for the graphic reproduction.

In the anterior regions a gliding movement of particles at the tip and the periphery of the pseudopod must be interpreted as evidence against a rolling movement of the plasmalemma, as well as against extensive new building of membrane in connection with locomotion, both phenomena requiring the particles at the tip to disappear. The only satisfactory explanation is given by the "gliding membrane theory": The whole plasmalemma glides over the stationary ectoplasmic gel tube with the same velocity as the advancing tip, similar to the finger of a glove. In connection with the folding and unfolding of the membrane, movement phenomena are more complex at the uroid than at the advancing tip: In the sequence from which the analysis in Fig. 2 was taken the uroid region was marked with active charcoal. In stage I–III the distance between particle a and b grows. During the following phase III–V the reverse phenomenon is found. This alternating change of distance between neighbouring particles can best be explained by alternating folding and unfolding of the cell membrane in this region. To test this explanation further, an adequate model was employed to imitate the movement of amoeboid cells. Folding and unfolding of successive areas of the uroid region can be simulated with a tin wire, and the resulting movement of marks on the wire shows the same characteristics as the movement seen with marker particles on the surface of the living cell (see also: Stockem, Wohlfarth-Bottermann and Haberey 1969).

Successive folding waves as used for the reconstruction in Fig. 3 would enable

the cell to move without alternating changes of length. Alternating shortening and elongation as proposed in Czarska and Grębecki's model were not substantiated by our observations so that the tin wire model may demonstrate the actual movement phenomena of the membrane.

Analysis of *Amoeba* moving on a horizontal ridge filmed in profile shows that surface contact is maintained only by small "micropseudopods" on the ventral side of the cell (MP in Fig. 4). In surface contact microscopy the contact areas at the tip of these small pseudopods can be made visible by interference technique (CA in Pl. IV 5) (Curtis 1964). By observation of the fringes a continuous oscillation of the contact areas is evident. This would indicate that surface contact at the single "micropseudopod" is only temporary, so that the forward gliding movement of the ventral plasmalemma is only temporarily and locally stopped. Local differences in gliding velocity must therefore be expected at the ventral cell periphery. However, this possibility remains to be demonstrated.

The difference between the membrane velocity at the dorsal and ventral cell surface which is evident by the relative change of positions of the particles A and B in phase I–VIII in Fig. 4 could be caused by the obstacles which local contact areas represent to the gliding forward movement of the plasmalemma on the lower side of the cell.

Discussion

Structures that may be responsible for the gel tube contraction have been found by recent workers in the form of thin fibrils and filaments (Schäfer-Danneel 1967, Wohlfarth-Bottermann 1960, 1963 a, b, 1964). Biochemical analysis identify these macromolecules as an acto-myosin-like protein complex (Simard-Duquesne and Couillard 1962 a, b). To explain the transformation cycle mentioned above at least three different stages of interaction of contractile proteins should be expected:

(1) fluid state: none or little rigidity of macromolecular connections (endoplasm);

(2) rigid state: rigidity of macromolecular connections (ectoplasm);

(3) shortening of the gel tube: a macromolecular contraction mechanism (ectoplasm in the uroid region).

Controlled movement as seen in phototaxis, for instance, is only possible when the temporal succession of these different stages follows an organized pattern and can be regulated by the cell. Though information about possible mechanisms for the regulation and control of contraction phenomena in protozoans are few, interesting results should be expected from further research carried out on Ca^{++} accumulating systems as found in muscle (Hasselbach and Weber 1965, Komnick 1969) and recently in slime mold plasmodia by Braatz and Komnick 1970. Further developments in the area of ion transport will certainly aid in the elucidation of the control mechanisms of the contraction cycle as well as to clearify the connection

of bioelectrical potentials along the membrane with ionic transport phenomena in protozoans (see Bingley 1966). Undoubtedly information about physical and chemical influences from the surrounding medium can be perceived by the cell, transformed into regulating signals and used to control the direction of flow.

That we now have obtained a model of the basis mechanism by which locomotion can be achieved in *Amoeba proteus* does not, however, solve the problem of amoeboid movement, but may help to clear up the remaining difficulties.

Summary

A combination of the results obtained by analysing microcinematographic motion picture films gives the following picture: During locomotion of *Amoeba proteus* the plasmalemma that covers the periphery in advancing areas of the cell moves freely over the ectoplasmic gel tube. In posterior regions the cell membrane is folded and constitutes a membrane reservoir for eventual plasmalemma demand in advancing regions. There is no active contribution of the cell membrane to the locomotion of *Amoeba proteus*. The plasmalemma is passively drawn over the ectoplasmic gel tube in advancing pseudopods and is again folded in posterior regions as a result of shortening and contraction of the ectoplasm at the uroid or in retracting pseudopods.

There is no evidence for a "fountain zone contraction" as put forth by Allen 1961 a, b, whereas all observations can readily be integrated into the "hydraulic pressure flow" hypothesis. According to this hypothesis the cell membrane surrounds a continuous closed transformation cycle: Contraction and simultaneous ecto-endoplasm transformation in the uroid region produces an endoplasmic pressure flow in the forward direction. The pressure is transmitted onto the plasmalemma at the tip of advancing pseudopods and as a result of this hydromechanical pressure the cell membrane is forced to move forward and at the same time to follow this forward movement by gliding over the ectoplasmic gel tube. After being deflected by the anterior membrane, endoplasm moves sideways to the periphery where it is transformed into ectoplasm and contributes to the elongation of the gel tube and thereby to the forward movement of the whole pseudopod. By the time it takes the *Amoeba* to move a distance equivalent to the length of the cell the formerly new built part of the gel tube is now located in the uroid and starts to contract again.

Continuous repetition of this transformation cycle causes permanent transport of volume in forward direction resulting in the forward movement of the cell.

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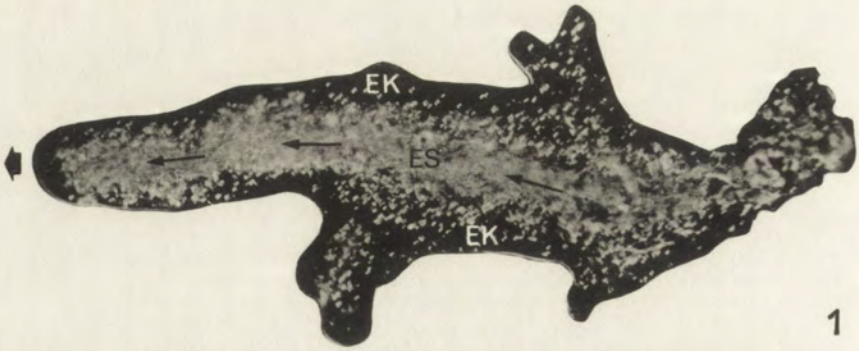
EXPLANATIONS OF PLATES I–IV

1: Normal moving form of *Amoeba proteus*. EK—ectoplasmic gel tube, ES—endoplasmic stream
2: a—tip of an advancing pseudopod. b—flow and transformation phenomena reconstructed from single frame analysis of motion picture films. EK—ectoplasmic gel tube, ES—endoplasmic stream, TZ—transformation zone
3: a—uroid region of polypodial *Amoeba proteus*. b—analysis of particle movement in the peripheric gel tube during uroid retraction. Successive stages I–VII have been recorded at 12.5 sec intervals. a, b, c and d—cytoplasmic inclusions
4: a–f—uroid contraction of polypodial *Amoeba proteus*
5: Interference contact microscopical picture of advancing (VP) and retracting (RP) pseudopods of *Amoeba proteus*. FR—frontal region



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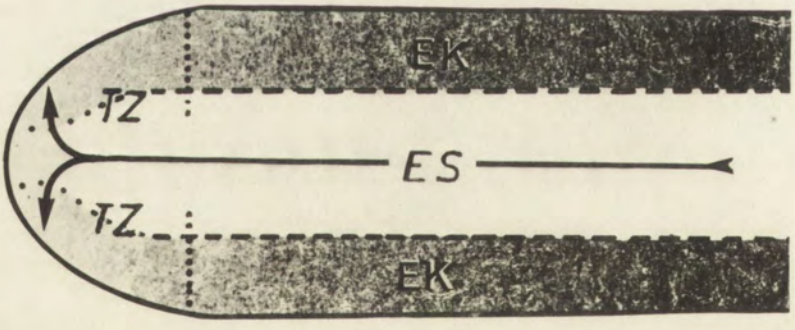
2a



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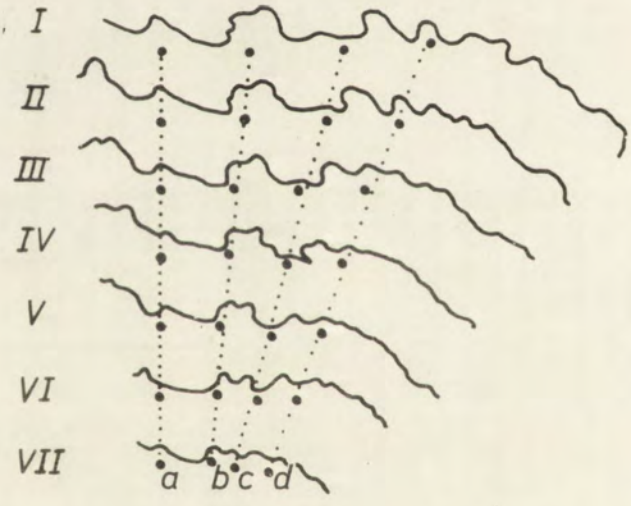

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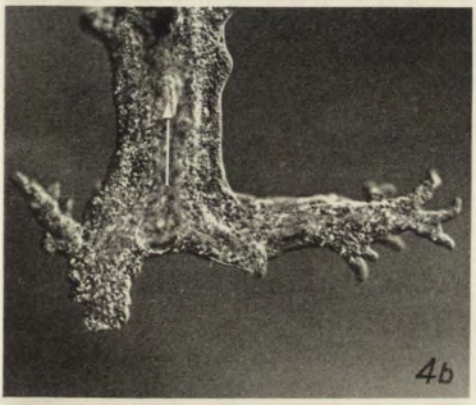

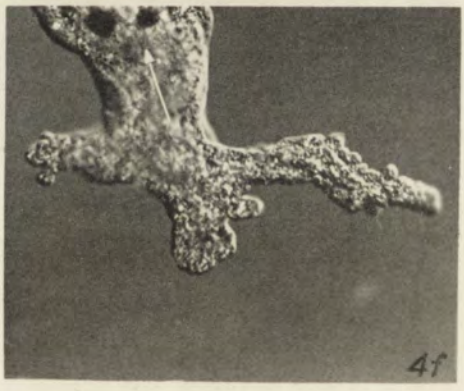

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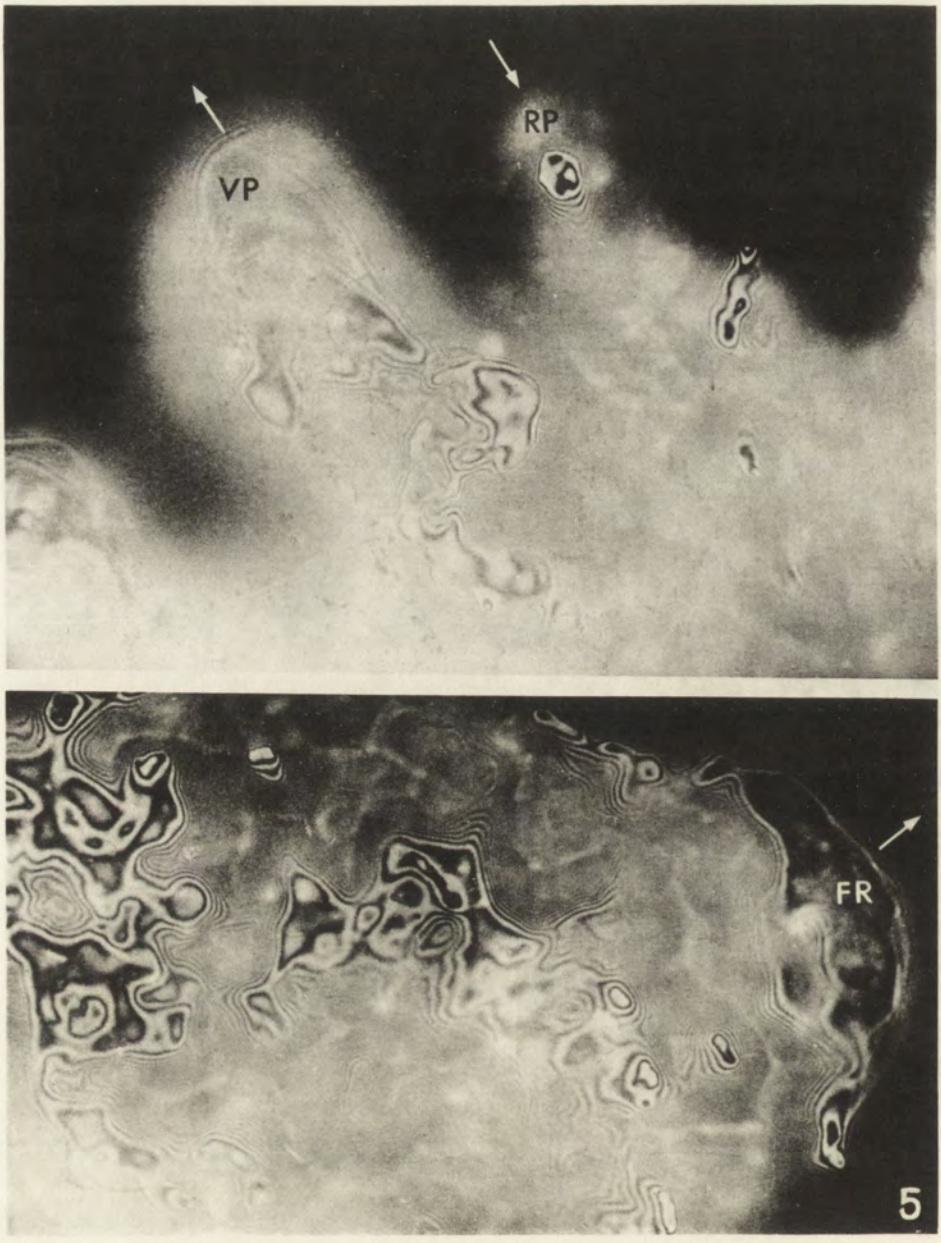

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Two movements in amoeba locomotion

In course of the last years successful work has been done to demonstrate and isolate filaments from amoeba cytoplasm and to show similarities with contractile proteins of the muscle. But it cannot be overlooked that the biological phenomena, which are performed by these structures are not yet explained. The ideas are even contradictory though for 135 years many efforts have been undertaken to understand the mechanism of amoeboid movement. The lack of this basic conception for the living amoeboid cell leads in consequence to uncertain interpretations of subcellular findings, both electron microscopic and biochemical.

It seemed generally agreed that amoeboid movement is due to the contraction of the cortical gel at the rear portion of the cell when Goldacre and Lorch (1950) extended the tail contraction hypothesis of Pantin (1923) and Mast (1926) to processes on molecular level. But Marsland (1964) presented a modification of this hypothesis because of the fact that any part of the plasma-gel may initiate contraction, and Allen (1961) on the other hand favors the view that propagated contraction occurs in the fountain zone. Amoebae can differ in respect to form changes, streaming intensity, etc., considerably. This demonstrates that several different types of amoeboid movement exist (Allen 1970). Nevertheless, the question concerning the fundamental mechanism which is common to all types of amoeboid movement including fibroblasts, leucocytes, mycetozoa, and others ranks before the problem of differences of secondary importance among the amoebae.

According to the fact that amoeboid movement includes cell locomotion, form changes and cytoplasmic streaming we are confronted with a rather complex problem.

There must exist a mistake in the reflection of the fundamental processes of this very dynamic type of movement. Otherwise it cannot be explained that in spite of intensive work we have no clear idea of this indeed complicated type of cell motility.

Our own investigations deal with the questions which especially concern the process of amoeba locomotion. The objects of our studies are the net-plasmodia of *Labyrinthula* and single amoebae which belong to the life cycle of these organisms.

We have found in accordance with Zopf (1892) that the net-systems of *Laby-*

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rinthula are composed of amoebae with fused hyaline ectoplasm and separated granulate endoplasm. But in the case of *Labyrinthula* net-plasmodia ectoplasm and endoplasm are separated from each other by a membrane system, though this separation is interrupted at several points by an organelle (Stey 1968, 1969; Porter 1969). Both parts – ectoplasm and endoplasm – are essential constituents of the free-living cell (Stey 1969, Schmoller 1971). This special morphological structure of *Labyrinthula* represents single amoebae during encystation (Schmoller 1966). The ectoplasm of the amoeba is going to form the envelope of the cyst around the endoplasm, fuses between neighbouring cells and thus represents the thread system of *Labyrinthula*.

Labyrinthula enables us to estimate the role of the ectoplasm and of the endoplasm in connection with the locomotion because they are separated (Schmoller 1971). Never before was this possible because of the dynamic organization of single amoebae.

How does move *Labyrinthula*? The locomotion of *Labyrinthula* net-systems includes a low-rate movement of the hyaline ectoplasm and intensive movement of the granulate endoplasm. Each of these two movements depends on the existence of the other. The endoplasmatic spindle-bodies of *Labyrinthula* slide in the fused hyaline ectoplasm. They get outside if the ectoplasmatic threads are destroyed and lose their ability to move. In the *Labyrinthula* net-system the endoplasmatic constituents of the cells cannot invade the utmost tip of the ectoplasm but become motionless some microns before. The lack of one or more factors which are necessary for the movement or the existence of one or more inhibitory or relaxing factors – counteracting biologically against the contraction – must be postulated.

The tip of the endoplasm plays an important role in its movement. It can be observed that it slides into a branch of the ectoplasm and later on takes the mass of it forward. The surrounding ectoplasm contracts at the rear portion of the endoplasm in the same degree as it is extended in the front, when a single spindle-body slides forward. But if there are masses of endoplasmatic spindles, 3–5 of them may be arranged side by side and the surrounding ectoplasm is broad. In this case no contraction of the ectoplasm takes place, which more clearly demonstrates that ectoplasm contraction is not a motive force.

The ectoplasm moves from backward to a stimulus (Aschner and Kogan 1959) and in addition it may be sent forward in course of directed cell growth. A sliding mechanism of molecular structures must be postulated. Ectoplasm alone, without presence of endoplasm cannot move but degenerates and falls to small droplets. The low rate of movement of the ectoplasm limits the locomotion of the whole cell system though the sliding rate of the endoplasm is by far higher.

In *Labyrinthula* there is no formation of pseudopodia which constantly change the shape. There is no free axial stream of the protoplasm, no ecto-endoplasm-process and no sol-gel-transformation. These phenomena are therefore unimportant in respect to its locomotion.

We transferred these results to the mechanism of amoeboid movement. The first point of this comparison is the opinion that continuous change of the cell shape and a free axial stream of the protoplasm are not correlated with amoeba locomotion. In other words; amoeboid movement which is characterized by everchanging of the cell shape, free streaming of the axial protoplasm and locomotion comprises several different mechanisms for these three phenomena. This result agrees with Seravin's hypothesis of multiple mechanisms in amoeboid movement (Seravin 1964, 1965, 1966, 1967). The next result from our comparison is the idea that amoeba locomotion results from the ecto-endoplasm-interrelationship as it is known from plant cells. But amoeboid movement differs for protoplasmic streaming in plant cells in so far as there is a functional centre in the tip of the endoplasm and the motive force is not set free along the ecto-endoplasm boundary. The result is locomotion, contrary to cyclosis in plant cells. Furthermore our studies show, that from ecto-endoplasm interrelationship there result sliding mechanisms of movement, with very low intensity in the case of the ectoplasm and with a high intensity in the case of the endoplasm. Whether we get the impression of slow or fast movement may depend only on the quantitative proportion of ectoplasm to endoplasm.

Ultrastructural and biochemical investigations on amoebae revealed fundamental relationship with the muscle. We expect that further investigations on amoeboid organisms will give new impulses in this field.

Summary

Comparative studies of *Labyrinthula* and single amoebae showed that amoeboid movement comprises locomotion, free axial stream of protoplasm and the changing of the cell shape with different mechanisms. Amoeba locomotion already involves a slow movement of the ectoplasm and a fast movement of the endoplasm and we agree with Seravin's hypothesis of multiple mechanisms in amoeboid movement. Sliding of molecular structures can be supposed to be a primary mechanism of amoeba locomotion.

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The role of external calcium in motile phenomena of *Amoeba proteus*

It has been observed (Pantin 1926, Mast 1928, Pitts and Mast 1934) that the shape and velocity of locomotion of *Amoeba proteus* depends on the ratio of monovalent and divalent ions in surrounding media but only slightly on the concentration of those ions. Especially, the main role of potassium versus calcium ions in ameboid motility has been noticed. Those data correspond with findings of Kamada and Kinosita 1940, carried out on *Paramecium*. Jahn 1962 restudied the data of Kamada and Kinosita in terms of the Gibbs–Donnan equilibrium and found that the duration of ciliary reversal is independent of the cation concentrations if $\frac{[K^{1+}]}{\sqrt{[Ca^{2+}]}} = \text{const}$. According to Jahn's 1962, 1967 hypothesis, the membrane of *Paramecium* works like a physical cation exchanger and changes of the amount of calcium adsorbed on the membrane are related to changes in duration of ciliary reversal. For *Paramecium* this postulate has been confirmed experimentally by Grębecki 1964, 1965, Kuźnicki 1966 and Naitoh 1968.

Furthermore, Jahn and Bovee 1969 suggested that changes in the Gibbs–Donnan equilibrium may affect the character and the rate of amoeba locomotion as in the case of ciliates.

The purpose of the present paper was to verify the role of calcium factor in amoeba movement.

Material and methods

The experiments were carried out on *Amoeba proteus* of S strain. Mass cultures were grown on Pringsheim solution with *Colpidium* sp. as a nutrient food. All test solutions were prepared with Tris-HCl buffer at pH 7.6. The solutions of the balanced salts $CaCl_2$ and KCl were prepared in the following Gibbs-Donnan values: 0.25, 1.0, 4.0,16.0. The final concentration of calciumchloride and potassium chloride amounts to about 4.5 mM, in all cases. The pH of all experimental solutions were adjusted to 7.6. As a control Tris-HCl buffer at pH 7.6 was used.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

To establish the influence of balanced salts on a rate of locomotion, the following procedure was performed. The amoeba, washed previously three times in Tris-HCl buffer, was transferred to a glass chamber which was filled with a test solution in the concentration mentioned above. After three hours the first solution was replaced with the second one of another Gibbs–Donnan value. The path traced by the amoeba was recorded macrophotographically in dark field illumination by means of a Robot camera. Every exposition lasted one hour, so for each Gibbs–Donnan value three pictures were taken. The lengths of paths were measured by means of a microfilm reader with a calibrated magnifier.

The process of decalcification in absence of calcium ions in surrounding media was studied at pH 7.6, such agents as KCl, EDTA and EGTA being used. Before the experiments the cells were also washed three times in Tris-HCl buffer to remove contaminating matter and ions present in the Pringsheim culture solution. The behaviour of amoeba treated with solutions of KCl, EDTA and EGTA as well as after the specimens had been washed with buffer or calcium chloride solutions, was observed by means of various microscopic techniques and photographed on 16 mm cinefilm. The movie-pictures were taken at a speed of 1.6 to 3.2 frames per second with interference-polarizing optics. The behaviour of amoeba as recorded on UK 18 ORWO-Color film. By projecting the film onto a piece of tracing paper by means of a film analysing projector all details of inhibition and restitution of locomotion manifested by changes of the amoeba shape were studied.

Results

The analysis of the paths covered by *Amoeba* revealed that whenever the specimen was transferred gradually from the lowest Gibbs-Donnan value (0.25) to the highest one (16.0), or backwards, no distinguishable changes in the rate of locomotion or character of paths covered by specimens were noticed. The same results were obtained for the specimens transferred directly from the lowest to the highest value.

On the basis of these observations further experiments on decalcification of *Amoeba* membrane were performed. The first experiments were carried out by means of potassium chloride of 10.0 mM concentration. The cessation of locomotion movement was noted after 2 h or more. Renormalization of locomotion after washing of amoeba with 10.0 mM Tris-HCl buffer lacking calcium ions was often observed.

The response of *Amoeba proteus* to chelating agents such as EDTA or EGTA in 1.0 mM concentration was the same – so in further experiments EGTA was used as a specific agent for calcium.

The response of the amoeba treated with EGTA was always similar. The first sign of response was a rapid stop of locomotion. Sometimes it was followed by backward streaming of cytoplasm. During next 10 min the shortening and strong folding of the membrane were observed (Fig. 1 A, Pl. I 2, 3). After this time the process was over, and the specimen was stopped in a typical shape similar to a pinocytotic rosette (Fig. 1 B, Pl. I 4). The contractile vacuole was also stopped. During this process a complete detachment from the substrate occurred. Despite a cessation of motion a strong cytoplasmic streaming still took place within the cell.

In those experiments a mechanical stimulus seems to be important because

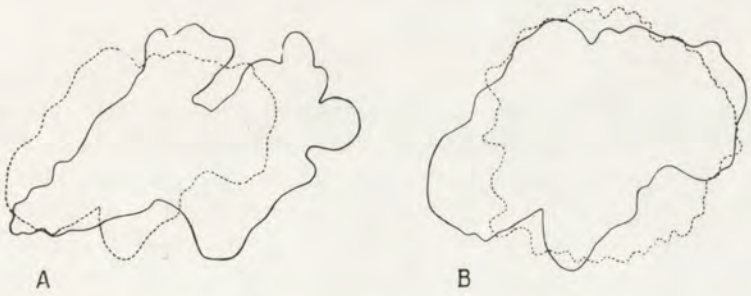

Fig. 1. The main stages of changes of amoeba shape after EGTA treatment. Consecutive stages of cease of locomotion of amoeba was drown by combining two stages — every previous shape has been drown by solid line while the broken line was used to express further stage. Figures 1 A and 1 B correspond with pictures 1, 2, 3 of Pl. I

the transfer of amoeba from one chamber containing the 1.0 mM EGTA to another with the same concentration of EGTA, revealed the beginning of a weak pseudopodial activity. A stronger reaction could be observed when the amoeba was transferred from the vessel filled with EGTA to another one with Tris-HCl buffer only; however, in this case the restitution of locomotion never occurred either.

The process of renormalization of the amoeba locomotion occurs after the adding of a calcium chloride solution (about 1.0 mM). Other salts like magnesium chloride and sodium chloride used in the same range of concentrations do not work the duration of the process of renormalization is from 10 to 30 min. At the beginning of this process the contractile vacuole starts working, the protoplasmic streaming is faster and short pseudopods appear in all directions (Fig. 2 A and Pl. I 5, 6). At least one of them becomes dominant and locomotion starts (Fig. 2 B and Pl. I 7, 8). The restitution of locomotion occurred when the amoeba was treated with EGTA for no longer than 10 min.

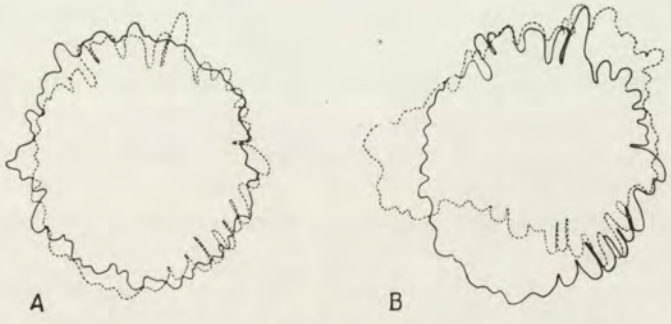

Fig. 2. The main stages of restitution of ameboid movement after adding of 1.0 mM $CaCl_2$. Figures have been drown in manner like on Fig. 1. Figures 2 A and 2 B corresponds with pictures 5, 6, 7 of Pl. I

Discussion

According to the Gibbs–Donnan equilibrium, changes of potassium–calcium ratio in studied concentrations have no influence on amoeba locomotion. So it seems rather impossible to control the rate of locomotion by means of different potassium and calcium concentrations. This confirms previous Mast's (1934) observations that the rate of locomotion is the same at pH 7.6 in solutions containing ions Na^+ and Ca^{2+}, K^+ and Ca^{2+}, and Ca^{2+} alone. By action of potassium ion (in concentration 10 mM) the locomotion of amoeba can be retarded or even stopped but:

(1) absence of Ca^{2+} in medium is required,

(2) the action of K^+ is very slow,

(3) the renormalization of locomotion occurs after washing in a buffer solution devoid of potassium ions.

Only chelating agents like EDTA or EGTA are able to stop the locomotion of amoeba immediately. The recovery of movement in a motionless amoeba occurs only after rinsing it in a solution containing calcium ions.

In the light of the data presented above, it is evident that calcium ions play a role in motile phenomena of *Amoeba proteus* but in a different way than in *Paramecium*. In *Paramecium*, changes in the level of calcium ions adsorbed on the plasma membrane are accompanied by depolarization of the membrane. Those facts indicate that in ciliates external calcium is a factor involved in excitation phenomena and liberation of calcium ions is a trigger mechanism promoting the ciliary reversal. In the amoeba, there is no evidence that changes in the level of calcium adsorbed on the external surface of plasma membrane influence the protoplasmic streaming. Probably the effect of chelating agents on the locomotion of amoeba depends only on changes on the surface during the removing of calcium involving lack of contact with a substrate and changes of charges on the surface. It is well-known that locomotion of amoeba depends on contact with a substrate. After removing the calcium ions from the membrane during the action of chelating agents the immediate retain of locomotion occurs with a characteristic detachment from the substrate. Generally, the role of calcium in contact phenomena has been discussed for other cells by Weiss 1962. Wolpert and Gingell 1968 suggested that the same calcium mechanism may play a similar role in ameboid motility. Simultaneously with decalcification, there occur some changes in charges on the surface of organism. The impedance of cell surface decreases and the amoeba ceases streaming and begins to exhibit pinocytosis (Brandt and Freeman 1967). It is in agreement with the presented observations that after EGTA treatment the amoeba settles into a shape resembling a pinocytotic rosette.

Renormalization of locomotion after the action of potassium occurs when this factor is removed from surrounding media. In the case of chelating agents EGTA and EDTA the addition of external calcium is necessary to restore the locomotion. Differences between both reactions require further studies.

Summary

The influence of the balanced salts of K^+ and Ca^{2+}, according to the Gibbs–Donnan ratio, on the locomotion of *Amoeba proteus* was investigated. In the studied concentrations, these are no distinguishable changes in the amoeba locomotion. In further studies, decalcification of amoeba membrane was performed by means of potassium, EDTA and EGTA. The chelating agents were more effective than potassium in cessation of locomotion. In the case of decalcification by the action of potassium, removing of this factor from the medium is sufficient to restitute the locomotion, while in the case of chelating agents the addition of external calcium is necessary.

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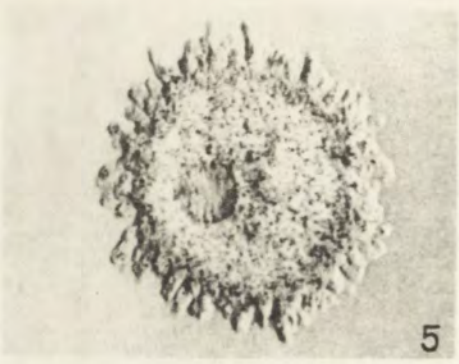
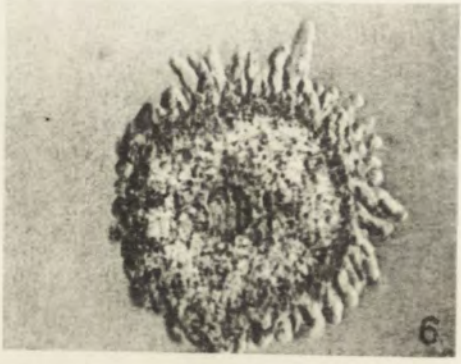
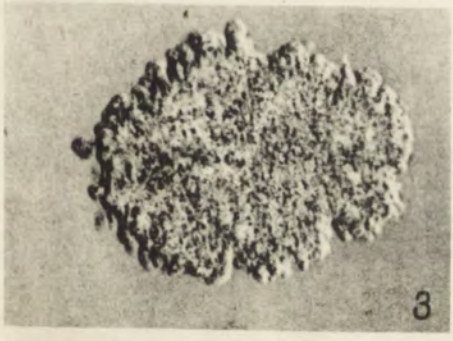
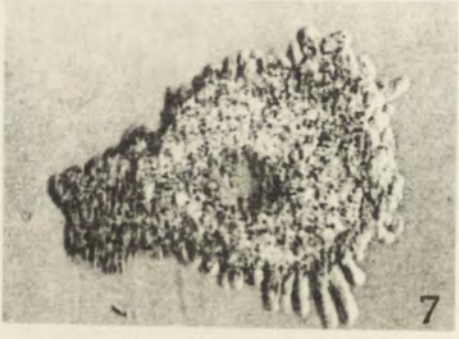
EXPLANATION OF PLATE I

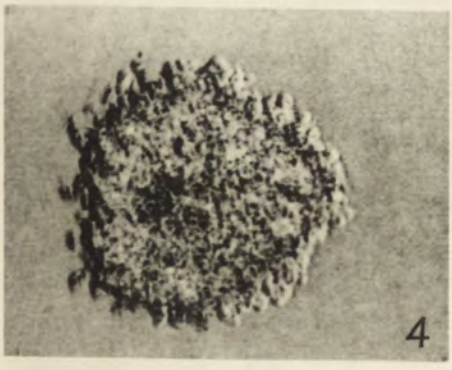
Consecutive stages of stopping and restitution of locomotion in *Amoeba proteus*
1: Normally moving amoeba
2: Cessation of locomotion after EGTA treatment
3-4: Folding of membrane and rosette shape appearance
5: Start of contractile vacuole pulsation afer adding of $CaCl_2$
6: Short pseudopods appearing in all directions
7-8: One of the pseudopods has become dominant and locomotion starts











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Contractile properties of the slime mold strand [2]

A segment of plasmodial strand is an exceedingly favorable material for analyzing the dynamic properties of a cytoplasmic gel. This is not because the material is only easy to handle, but also because the contractile force produced in a single direction can be measured. It is known that a piece of plasmodial strand of *Physarum polycephalum*, when isolated from the stock culture and hung in moist air, contracts and relaxes rhytmically with a period of 1.5–3.0 min, i.e. with the same period as that of cytoplasmic streaming. The contraction is about 1/10 the total length.

The present study was conducted in part at the State University of New York at Albany in 1970 and continued more recently at Osaka University. Our aim was to elucidate the dynamic characteristics of the slime mold strand by measuring (1) spontaneous changes in tension while the length of the strand was kept constant (isometric contraction), and (2) spontaneous changes in length keeping constant the tension applied to the strand (isotonic contraction). Torsional movement (Kamiya and Seifriz 1954, Kamiya 1965), also a remarkable characteristic of this material, has not been taken into account here.

Material and methods

Physarum polycephalum was cultured by the method of Camp (1936) on moist filter paper covering an overturned petri dish. Outgrowths of the plasmodium over the wall of the container of the stock culture were sampled for the segments of plasmodial strand used. These were lifted by forceps and mounted directly on the measuring apparatus.

Contractions of the plasmodial strand were measured with a sensitive tension transducer by holding the strand at both ends either in a vertical position in the moist chamber or in a horizontal position in water.

For the vertical system (Fig. 1) a tension transducer with a measurement range of 0–35 mg was constructed by converting a conventional microammeter into an electrobalance (EB). The

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[1] The work reported here was initiated while the senior author was a Senior Foreign Scientist award from the National Science Foundation, and was supported by a Program Grant GM-14891 from the National Institute of General Medical Science.

[2] A preliminary report appeared in Proc. Japan Acad. 46, 1026–1031, 1970.

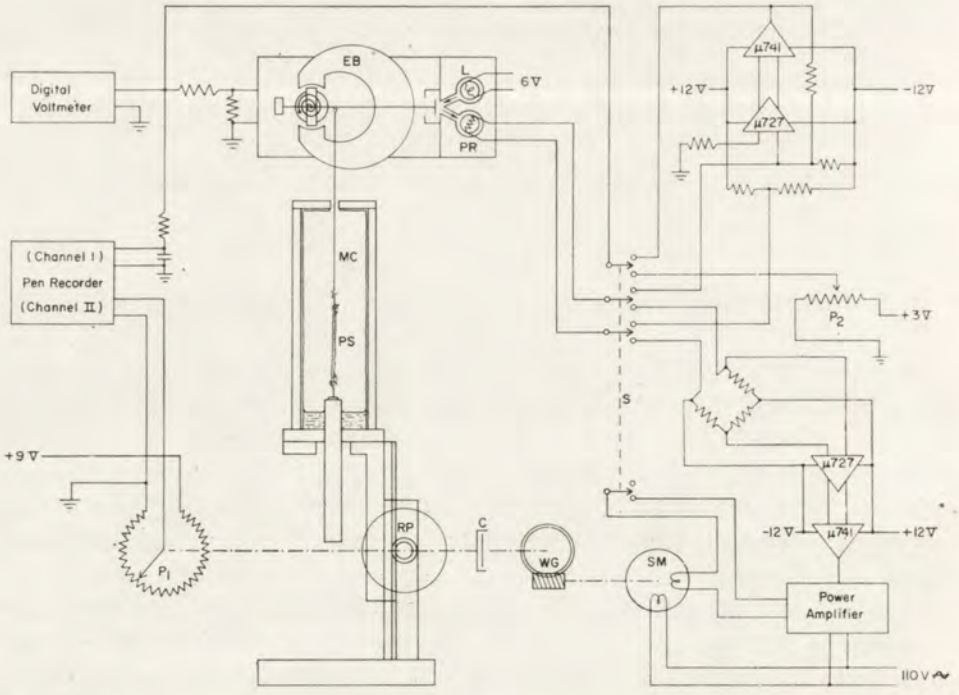


Fig. 1. Block diagram of the vertical system for measuring isometric and isotonic contraction in the hanging plasmodial strand. For explanation, see text

indicator hand of the microammeter, which serves as an arm of the electrobalance, is equipped with a tiny mirror reflecting the light beam onto a photoresistor (PR). The construction of the whole setup is such that either isometric or isotonic contraction can be measured alternatively by setting a compound switch (S) in the proper position.

For the measurement of isometric contraction, where the switch (S) was set on the upper connections, the arm of the electrobalance was kept at a constant horizontal position irrespective of the tension produced in the plasmodial strand. This was done by means of the photoresistor and feedback circuit which adjusted the supply of the current to the electrobalanace automatically. This adjusting current, which was a direct measure of the tension produced in the plasmodial strand, was registered on a chart with a pen recorder.

The maximum vertical shift of the arm of the electrobalance with a load from 0 to 35 mg was less than 0.15 mm, or less than 0.5% the total length of the plasmodial strand (PS) 30 mm long.

For the measurement of isotonic contraction the switch (S) was set on the lower connections. To impose a certain tension on the strand we applied a calibrated constant current to the electrobalance through potentiometer P_2. Information from the photoresistor (PR) was amplified to drive the servomotor (SM) in one direction or the other. The servomotor was connected with the rack-pinion mechanism (RP) by a worm gear (WG) and clutch (C). Thus the servomotor moved the moist chamber (MC), to which the plasmodial strand (PS) was fixed at its lower end, either upward or downward so that the position of the arm of the balance, and hence the tension of the plasmodial strand, remained always the same. The actual displacement of the

chamber (i.e., the change in length of the strand) was recorded as potential change through potentiometer P_1, the shaft of which was connected mechanically with the rack-pinion (RP) mechanism.

In the vertical system the tension in the strand is greater at its upper part than its lower part due to the strand's own weight. This is, however, not the case of we keep the strand in a horizontal position immersed in water. Another merit of the horizontal system is that it allows us to test the effects of various chemicals in the solution in which the strand is submerged.

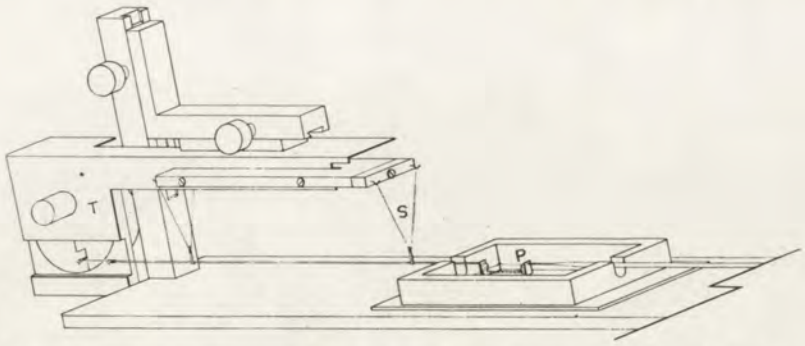


Fig. 2. Diagram of the horizontal system for measuring slime mold strand contraction. For explanation, see text

The horizontal system (Fig. 2), the plasmodial strand (P) is held in water between two small wooden blocks ($1 \times 2 \times 3$ mm^3), each loaded with a tiny mass of the plasmodium. The lower parts of the two wooden blocks are in water in a flat vessel made of methacrylate resin. Tiny plasmodial masses creeping on a pair of wooden blocks are mostly in the air, but the lower parts where the plasmodial strand joins them are in water. One of the wooden blocks is cemented to a solid glass rod affixed to a rack-pinion mechanism so that the strand can be stretched when desired. The other wooden block is attached to a delicate glass rod suspended horizontally with a pair of very fine V-form glass fibers (S). With this system the delicate horizontal rod is movable only in the longitudinal direction with least friction. This suspended bar is connected with the tension transducer (T), the construction of which is about the same as the one described before. Figure 3 shows a photograph of the horizontal system set on the stage of the polarizing microscope. So far, we have used the horizontal system only for measuring isometric contraction, not isotonic contraction.

Results

Isometric contraction

Figure 4 shows an example of a regular wave pattern of tension changes while the length of the strand is kept constant. Here the waves have a period of 1.6 min and a peak-to-peak amplitude of 5–6 mg. The maximum and minimum tensions developed are about 13 mg and 6 mg, the midpoint (not shown) being shifted slightly. Since the average diameter of the strand was 220 μm in this specimen, the tension developed is known to have oscillated within the range of 35 g/cm^2 and 18 g/cm^2.

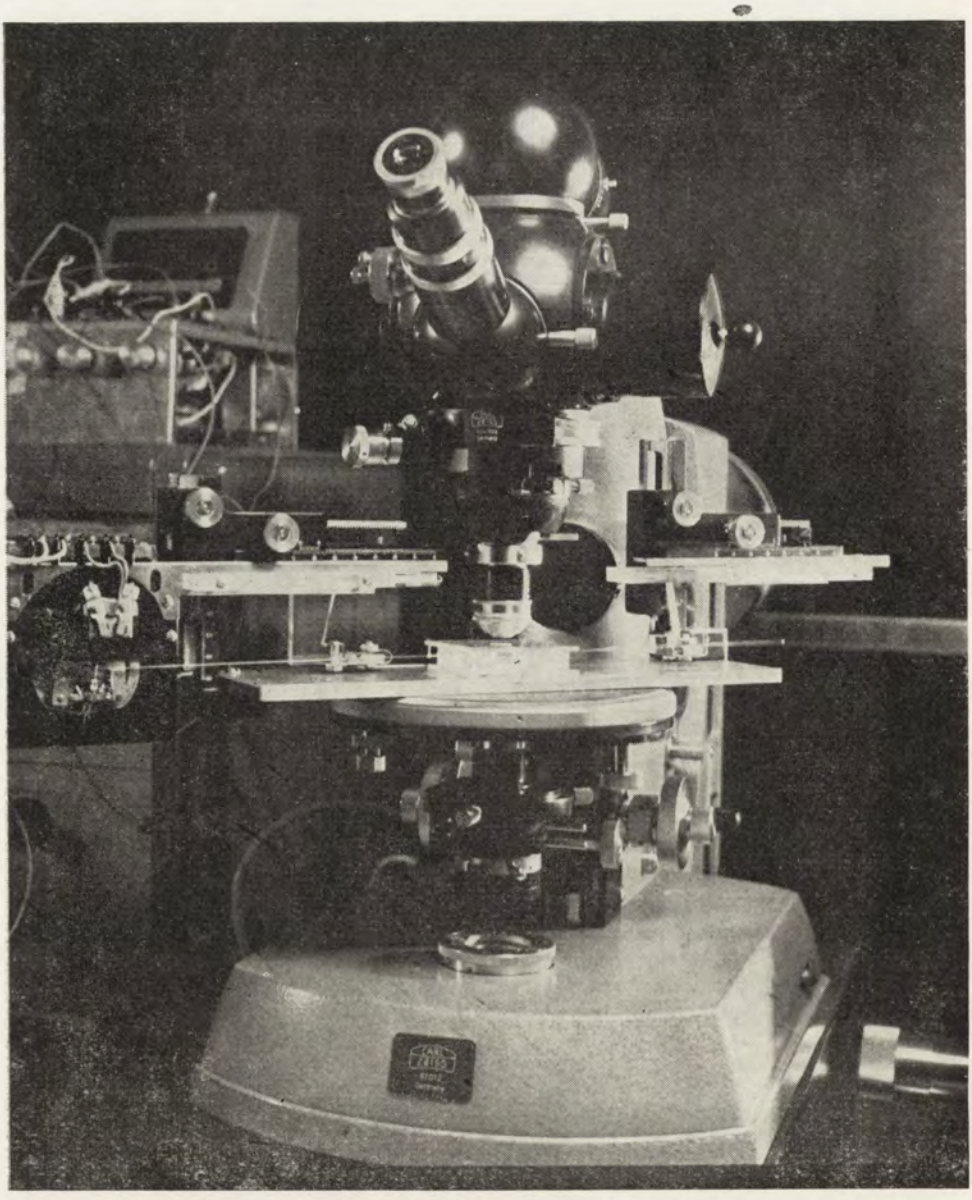


Fig. 3. Photograph of the horizontal system. See text

Figure 5 shows another example of isometric contraction keeping the strand with the length of 8.2 mm and the width of about 400 μ in a horizontal position. It is noticed here that all the waves having a period of about 3 min show a similar pattern with a conspicuous shoulder on the increasing-tension phase.

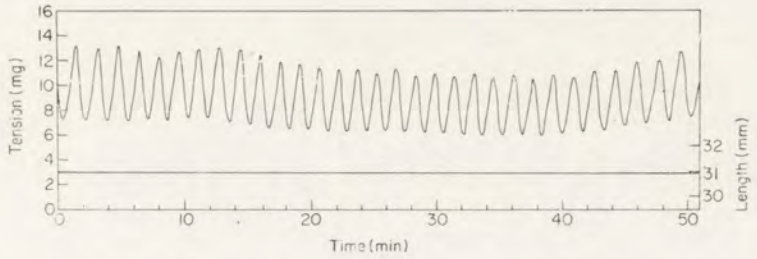


Fig. 4. Tension (in mg) as a function of time (min) during spontaneous contractions of a plasmodial strand segment

Figure 6 represents a similar case of isometric contraction of a horizontal strand, 16.5 mm long. Again, a shoulder appears on the increasing-tension phase. Under isometric conditions, there were some changes in the diameter of the strand, but they had no correlation with the tension developed.

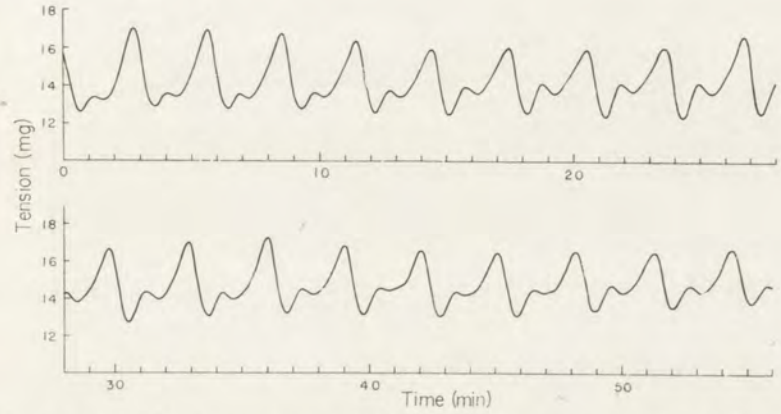


Fig. 5. Same as Fig. 4. Note conspicuous shoulders in the increasing tension (contractile) phase of the cycle

In Figure 7 a short strand was stretched stepwise. It was noticed that whenever it was stretched by 20–27% of the total length, tension increased immediately. The midpoint of the waves (not indicated), however, came down rather rapidly at first and less rapidly afterwards showing tension relaxation each time. A fact to be stressed here is that not only the absolute value of tension, but the amplitude of the tension wave increased tremendously on stretching in spite of the fact that the strand became thinner at each stretch. The average diameter after the second stretch (40–70 min) was 260 μm in this case. The amplitude once increased on stretching, however, started decreasing again as the tension decreased. This was true of all the cases examined. There was, however, a tendency for the increase in amplitude to disappear if the strand was pulled too much (cf. the 3rd and 4th pulls in Fig. .7)

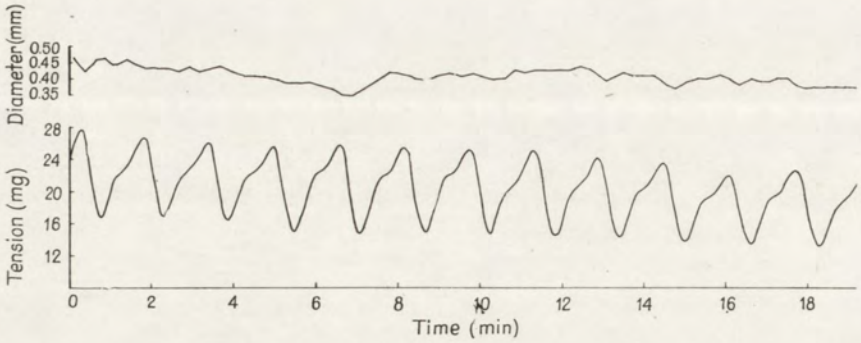


Fig. 6. Lower curve: same as Figs. 4 and 5. Upper curve: changes in diameter with time

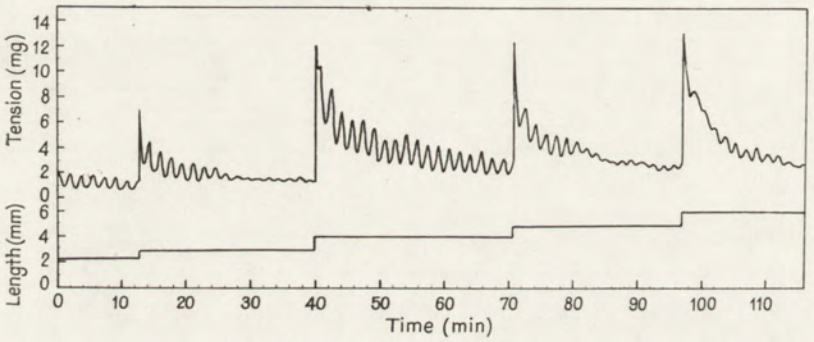


Fig. 7. Increases in the amplitude (tension) of isometric contraction in a plasmodial stran exposed to step-wise stretching

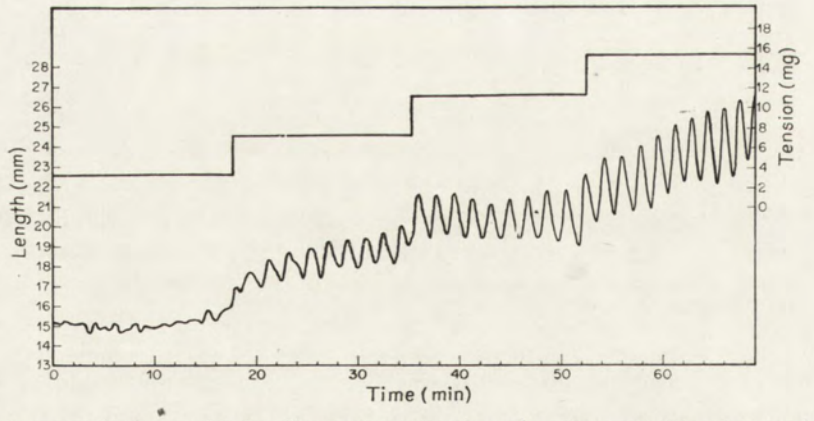


Fig. 8. Increases in the amplitude of isotonic contraction when the applied tension was increased in steps

Isotonic contraction

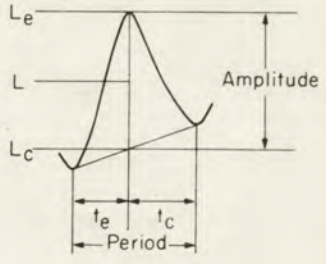
Figure 8 shows an example of isotonic contraction where the strand was loaded stepwise with increasing tension. Soon after the strand was hung in the moist air with a moderate load (3.4 mg), the waves became irregular in form and insignificant in amplitude. As the tension was increased stepwise by 4 mg, i.e. to 7.4 mg, 11.4 mg, 15.4 mg, the amplitude of the wave increased also stepwise and the wave form became regular. The cyclic contraction waves at different tension levels, however, did not tend to decrease their amplitude. This is a good contrast to isometric contraction where the amplitude of the tension waves decreased remarkably as tension relaxation of the strand occurred. However, just as the increasing-tension phase in isometric contraction lasted longer than the decreasing-tension phase, the contraction phase here always lasted longer than the elongation phase. As in isometric contraction, a shoulder also appeared frequently during the contraction phase of the wave.

The numerical data from this record are summarized in Table 1. A further remarkable fact to be noticed here is that not only did the amplitude of the contraction wave increase under higher tension, but the rate of contraction also increased.

Table 1

Isotonic contraction of plasmodial strand under different tension levels

Load (mg)	7.4	11.4	15.4
Period : $t_c + t_e$ (sec)	110	110	100
Duration of contraction phase : t_c (sec)	64	66	57
Duration of elongation phase : t_e (sec)	46	44	43
t_c / t_e	1.39	1.50	1.33
Amplitude : $L_e - L_c$ (mm)	1.3	2.2	3.0
Specific amplitude : $(L_e - L_c)L^{-1}$ (%)	7	10.7	12.8
Rate of contraction : v_c ($\mu \cdot sec^{-1}$)	22	33	55
Rate of elongation : v_e ($\mu \cdot sec^{-1}$)	35	50	80
Specific contraction rate : $v_c \cdot L^{-1}$ ($\% \, sec^{-1}$)	0.12	0.16	0.23
Specific elongation rate : $v_e \cdot L^{-1}$ ($\% \, sec^{-1}$)	0.19	0.24	0.34



This is true in terms of what may be referred to as "specific contraction rate", defined as the per cent contraction per unit length of the strand. This is all the more to be stressed, since, to understand this phenomenon, we have to postulate some regulatory process in the plasmodium which augments the contractile force under applied tension.

Figure 9 shows an example in which isometric (top) and isotonic (bottom) contraction were measured alternately in one and the same strand. It is clearly seen here,

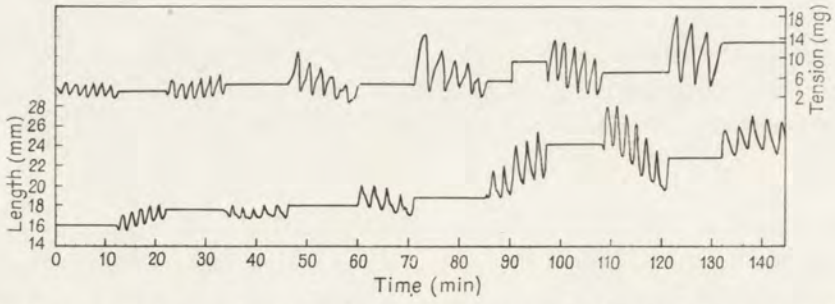


Fig. 9. Alternating measurements of isometric (upper record) and isotonic (lower record) contraction of a slime mold strand subjected to step-wise changes in length

too that the increasing-tension phase in isometric contraction (top) and contracting phase in isotonic contraction (bottom) lasted longer than the decreasing-tension phase (top) and elongation phase (bottom), and that the shoulders often appeared in the former phases. There has been practically no exception to this tendency.

Modulus of elasticity

The methods applied to the present work provided us with a good opportunity to study the modulus of elasticity of the plasmodial strand. Figure 10 is a photo-

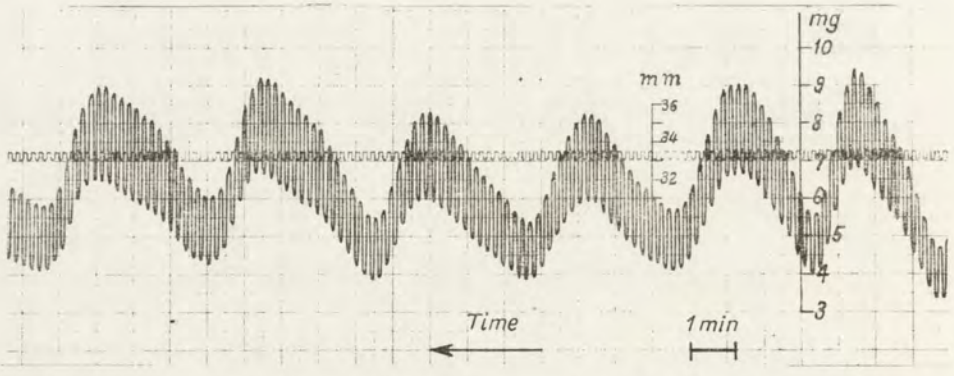


Fig. 10. Changes in tension of a spontaneously contracting strand while its length was changed by 0.5 mm each five sec

graphic reproduction of a record showing changes in tension of the strand when its length was modified alternately between 33.0 mm and 33.5 mm or by 1.5%. Each length lasted for 5 sec so that changes in length show symmetrical square waves with a period of 10 sec. From this figure, we notice that the vertical width between the upper and lower envelopes of the minor waves is the greatest at the peak and smallest at the trough of the major waves. Since the strand was kept nearly the same in diameter at ca. 200 μm, this record shows that the apparent Young's modulus has changed by about 30–40% according to the phase of tension production. The maximum and minimum values of apparent Young's moduli are about 5×10^5 and 3×10^5 dynes/cm^2 in this case. These values are, however, concerned with instantaneous elasticity followed by decay in tension. A further analysis of visco-elastic properties of the plasmodial strand will be reported elsewhere.

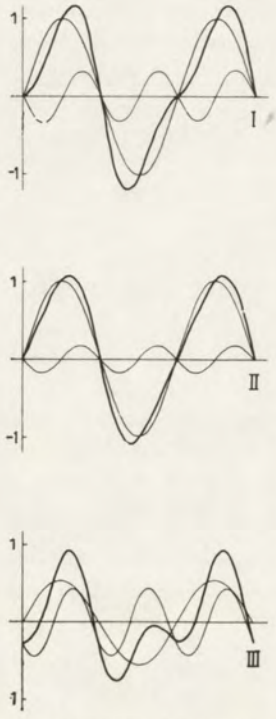
Discussion

The values of Young's modulus obtained in the present work is in the same order as those obtained by previous workers (Norris 1940, Abe 1965). Our special intention was, however, to correlate changes in Young's modulus with changes in spontaneous tension production. When strands are hung for a while with a load in a moist chamber, the value of Young's modulus gradually increases from 1×10^5 dynes/cm^2 or from even lower level to about 3×10^5 dynes/cm^2. This may reflect reorientation, under tension, of the cytoplasmic microfilaments observable electron microscopically from transverse to longitudinal direction of the strand (Kamiya 1968). On contraction, Young's modulus further increases to about 5×10^5 dynes/cm^2. This result may be a physical manifestation of the increase in cytoplasmic microfilaments observed by Wohlfarth-Bottermann (1964) during the contracting phase. However, it should also be noted that similar changes in elasticity occur during muscle contraction (Buchtal and Sten-Knudsen 1950).

The fact that the amplitude of isometric contraction depends on applied tension may have a profound significance for the molecular events which underlie these dynamic characteristics of the slime mold cytoplasm. This result implies that the contractile mechanism can somehow "sense" the applied tension and cause the redeployment or de novo assembly of more contractile material. The same events may occur during the contractile cycle as evidenced by the increase in elastic modulus. The simplest explanation for the effects of tension is that the degree of microfilament orientation is enhanced by tension. It has even been suggested by Francis and Allen (1972), who have measured strain birefringence in amoeba endoplasm, that tension applied to a more or less isotonic cytoplasmic gel might not only make it birefringent but increase the efficiency of contraction by orienting its contractile machinery.

We should also like to make a suggestion which might help us understand why the increasing-tension (contracting) phase lasts longer than the decreasing-tension

(elongating) phase and why a shoulder appears often in the contracting phase. A possible explanation is the presence of at least two different periods of contraction-relaxation cycles, where one period is close to one half of the other. This possibility is u ggested by the fact that most wave forms of isometric as well as isotonic contraction may be reconstructed closely enough with a sine wave and its 2nd harmonics having the proper amplitudes (Fig. 11). There must be, however, a definite phase relation between the two waves, so that the contracting phase can last longer than the relaxing



$$A = A_1 \sin(\omega t - \theta_1) + A_2 \sin(2\omega t - \theta_2)$$

	A_1	A_2	θ_1	θ_2
I	1	$\frac{1}{3}$	0	π
II	1	$\frac{1}{6}$	0	π
III	$\frac{16}{30}$	$\frac{13}{30}$	0	$\frac{9}{10}\pi$

Fig. 11. A few examples of reconstructed complex waves resembling those frequently observed cf. Fig. 5 and 6) by adding second harmonics of appropriate amplitude and phase to a fundamental sine waves

phase or that a shoulder appears only in the contracting phases. That is, the phase of the second harmonics is shifted by about π, from that of the fundamental oscillastion, or

$$A = A_1 \sin(\omega t - \theta_1) + A_2 \sin(2\omega t - \theta_2),$$

where $\theta_1 - \theta_2$ is found in the range of $(1/2 - 3/2)\pi$, mostly close to π, A_1 and A_2 are the amplitudes (in the sense ordinarily used) of the fundamental oscillation and 2nd harmonics respectively, and ω the angular velocity or $\frac{2\pi.}{T}$ Whether a shoulder

is more or less conspicuous may be due simply to the magnitude of the second harmonic relative to that of the fundamental wave.

Combination of the fundamental oscillation with its second harmonics at a definite phase relation may have a significant physiological meaning as a regulatory mechanism. If so, we have to look for what might mean at the molecular level.

So far we have limited our argument to some basic, physical aspects of contractility. The measurement of contractile tension in the plasmodial strand is essentially similar physiologically to the measurement of the motive force responsible for endoplasmic streaming in the same organism (Kamiya 1959). There are, however, two big technical advantages in the tension measurement over motive force measurement. First, the contractile force can be made unidirectional, and second, the condition of the material can be controlled more exactly. It will be important physiologically to test the response of the strand against physical and chemical agents. Ultrastructural research in connection with tension production is also exceedingly important. These problems have occupied our attention for a number of years (cf. Kamiya 1968, for other recent work) and many are still to be dealt with.

Summary

(1) Slime mold strands can develop tension periodically with maximum value around 50 g/cm^2.

(2) In isometric contraction, the amplitude of cyclic changes in tension increases enormously on stretching the strand.

(3) After stretching, relaxation of tension takes place rapidly; simultaneously the amplitude of the tension waves decreases.

(4) The increasing-tension phase lasts longer than the decreasing-tension phase in isometric contraction. Similarly, the contraction phase lasts longer than the elongation phase in isotonic contraction.

(5) There is a general tendency for a shoulder to appear in the increasing-tension phase of isometric contraction, as well as in the shortening phase of isotonic contraction.

(6) The apparent Young's modulus is of the order of 10^5 dyne/cm^2 and varies according to the phase of the contractile cycle. As expected, in the contraction phase the Young's modulus is larger than in the relaxing phase by ca. 50%.

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A detailed explanation of the distributed solation hypothesis and its application to slime mold plasmodia

The vegetative phase of the acellular slime mold, the plasmodium, exhibits a type of protoplasmic streaming in which the inner sol area first streams in one direction for approximately 90 sec after which it stops and reverses its flow. The flow continues to reverse direction at approximately 90 sec intervals. The direction of net movement of the plasmodium is controlled by the formation of an advancing front region and the majority of ionic uptake occurs in this area (Miller, Abbott and Anderson 1968). Depending upon the substrate composition, the mold migrates at different rates. In a starved condition the mold migrates rapidly (ca. 30–50 mm/h) whereas if substances such as 0.5 M glucose were in the substratum, the rate of migration would drop to 1.0 mm even though the internal protoplasmic movement remained. Thus it seems that there is (1) recognition of various substrates by the plasmodial system at the advancing front (2) a change in rate of movement at the advancing front if the right component (s) are present in the substrate, and (3) a change in morphology of the advancing front which is concomitant with the change in movement.

These findings prompted Rose, Miller and Anderson (1970) to look for a biochemical basis for its glucose inhibition of the migration phenomena. They found that *2*-deoxyglucose, a specific inhibitor of glucose-*6*-phosphate dehydrogenase was ineffective in interferring with either the rapid migration during starvation (starvation rate) or the reduced migration rate when glucose (feeding rate) was present in the substratum. In addition, fructose in the substratum would not inhibit migration nor would mannose, yet the mold would grow well with mannose as a carbon source. This plus other findings prompted them to postulate that the key factor in the glucose recognition by the mold was a glucose oxidase enzyme. In 1970 Miller and Reed outlined a Distributed Solation Hypothesis (DSH) to explain protoplasmic movement. The DSH included the following general concepts: sol spontaneously forming a gel, enzyme dependent solation of the gel, synchrony induced in solation elements by limiting compounds, and weak forces dependent upon volume increase

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and constriction of gel. The purpose of this work is to provide a detailed example which would fit the DSH as it applies to slime mold plasmodia.

Consider then, that the slime mold operates metabolically in two different modes – an exophagous mode (feeding from external sources) and an endophagous mode (feeding on itself). In the latter mode (see Fig. 1) cytoplasmic enzymes convert gel to sol and in the process produce precursors which are put together to form hexose.

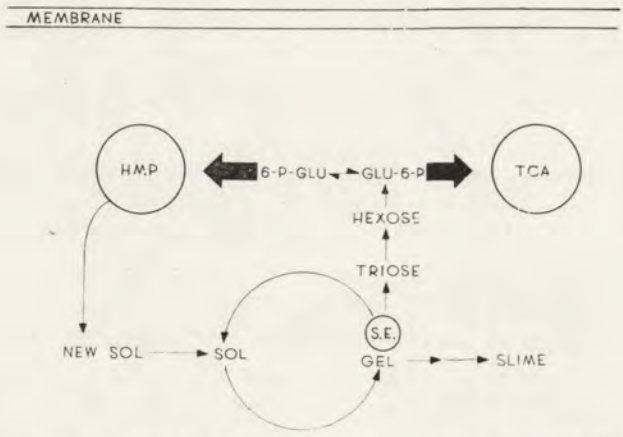

Fig. 1. Diagram of proposed endophagous process in slime mold plasmodia

The hexose is then converted to glucose-*6*-phosphate. Small amounts of the glucose-*6*-phosphate form *6*-phosphogluconate, but most of it is converted to fructose-*6*-phosphate. The small amount of *6*-phosphogluconate formed is utilized in the hexose monophosphate pathway (HMP) which provides a key factor for the formation of new sol. This sol ages, forming a weak gel first, which continues to constrict, forming a tighter gel as it ages. The process of gelation, accompanied by constriction of the gel network, is dependent upon the availability of calcium ions. Cytoplasmic enzymes may act upon the gel to convert it to sol and thus begin the endophagous solation process anew.

The fructose-*6*-phosphate derived from the endophagous process is metabolized via the TCA pathway and results in the production of ATP. The ATP thus formed first reaches molecules capable of a conformational change which are present in the endoplasm (weak gel) and they temporarily contract. This contraction is sufficient to break the very weak gel structure and convert it to a more fluid condition. The remainder of the ATP attaches to contractile molecules (microfilaments) in the gel structure of the ectoplasm and causes a transient contraction which will constrict the walls. Hence the presence of ATP in a given area of the endoplasm first lowers the viscosity of the endoplasm and next constricts the diameter of the tube which moves the solated element. A series of solated elements in a starving plasmodium

act like a series of resonating oscillators at equilibrium that develop a synchrony of protoplasmic movement and support rapid migration. While many of the components of the system may have periodic rhythms, it is presumed in this system that ATP is the limiting compound for synchrony. Hence in the autophagous mode, enzymes continually digest ectoplasmic gel to produce sol plus a hexose precursor which is used to synthesize ATP, and a minimal amount of sol. The organism is essentially digesting itself, becoming smaller, and migrating rapidly.

In the exophagous mode, (see Fig. 2) oxidase receptors which are glucose spe-

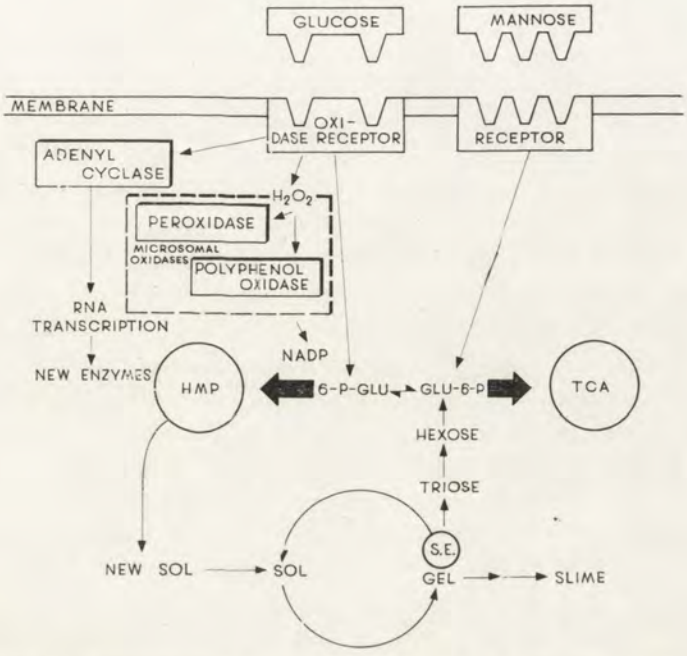

Fig. 2. Diagram of proposed exophagous processes (feeding from external sources) in slime mold plasmodia

cific phosphorylate glucose to produce 6-phosphogluconate which is metabolized principally by the hexose monophosphate pathway (HMP). Microsomal oxidases are a most important adjunct to this process since they provide a key factor – NADP – which allows the HMP to proceed at a rapid rate. This pathway provides a key factor for sol formation and in addition if the oxidase receptor operates, RNA transcription is activated via cyclic AMP to produce additional enzymes. In this mode the ATP produced is used principally in synthetic processes and to support the solating system. Very little if any at all is left over for attachment to contractile elements in the tube walls. Hence upon meeting proper substrate, sol formation increases, net synthesis increases, volume increases, motive force decreases, and the anterior

portion of a plasmodium literally grows over the surface. Nevertheless, the more rearward areas continue the rapid streaming.

The motility of a plasmodium is thus dependent upon two mechanisms of force development. First short range forces are developed by the solation elements in the act of solation. In this process, small increases in volume occur and are important in the formation of pseudopods and advancing front areas. Superimposed upon this basic mechanism may be longer range forces developed by contractile molecules. In order to be effective for motive force development, the contractile elements would have to be enclosed in gel. Contractile molecules present in the weak gel of the endoplasm would only help to break down the structure of the weak gel.

Discussion

Clearly the model implicates the metabolism in the generation of ATP which supplies the energy for the events which support the shuttle streaming, the HMP pathway is involved in the synthesis of sol. Specific oxidase receptors along with microsomal oxidases are involved in an increase in the synthesis of new sol and the production of new enzymes. Thus compounds which suppress the TCA pathway decrease ATP and thereby decrease migration. Those things which decrease the HMP pathway without affecting the TCA pathway would increase migration. Substances which specifically affect the oxidase system would not immediately affect the plasmodium, but because formation of solation enzymes would be inhibited, it would eventually bring the system to a halt. Defects of this type may also be genetically influenced and account for the plasmodium which loses its ability to digest vacuolar material, lives for awhile via autophagous metabolism, and eventually dies. A somewhat similar condition occurs in the chronic granulomatous disease of white blood cells.

The role of ATP in this hypothesis is multiple. Produced by a region of the endoplasm it causes a transient contraction of molecules embedded in the gel wall thereby temporarily limiting the diameter of the gel tube and forcing the movement of the solation element. If synthetic processes are competing for ATP, then the concentration does not get high enough to diffuse out to the ectoplasm and cause a transient contraction of molecules in the gel. Accordingly, substances or processes which raise the ATP concentration or stimulate its production increase the long range forces and those factors which limit ATP concentration without halting metabolism reduce the long range forces.

Calcium is important for the contractile elements but even more important when it attaches to protoplasmic molecules to promote the gelation process. Thus any process which sequesters calcium would promote the sol condition. In this respect, it is important to point out that although each molecular species is mentioned with respect to a single function, there is nothing to preclude a single molecule from

being multifunctional. Consider the enzyme diagramatically shown in Fig. 3. Located on it are several points of allosteric attachment.

Assume that the molecular portion which binds certain items can protrude through vacuolar membranes. The points of attachment of different items on the molecule are indicated by the different shapes (square, triangle, circle, etc.). It is assumed that the enzyme has a priority for binding to the various sites (Koshland 1958) and that when certain sequences of sites are satisfied, portions of the molecule can undergo a conformational change. For instance, in the diagram, if the sequence

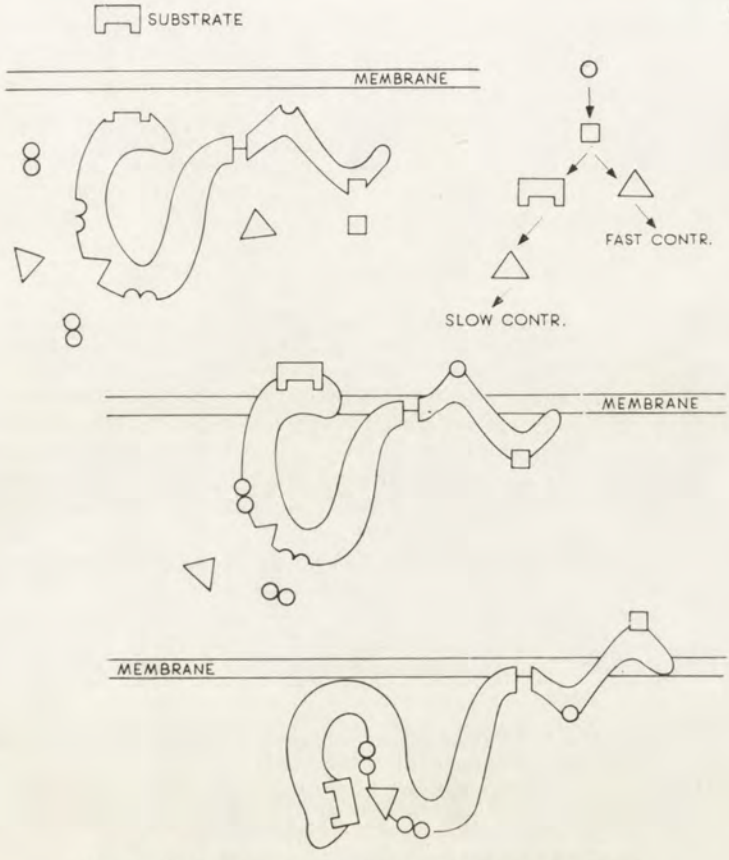

Fig. 3. Diagram of hypothetical multifunctional enzyme

circle, square, figure eight, triangle, and figure eight bind on the enzyme, then the right hand portion of the molecule twists and after enzymatic action all constituents are released from the sites and the molecule returns to its original shape. If, however, the comb shape representing substrate binds after the sequence circle, square, figure eight, a large change in shape (contraction) occurs in the left hand portion and afterwards the smaller twist occurs in the right hand portion of the molecule. Again after

the enzymatic action is complete, the components are released and then the molecule returns to its original shape. This is a generalized model of a cellular component which has several functions. Firstly, it is contractile and may be considered as a morphoplastic system. Secondly, if we assume that the triangle represents ATP then this molecule is an ATPase. Thirdly, if the circle is potassium and the square is sodium, then the enzyme is an ATP dependent transport system. If the substrate shown is glucose, then the enzyme is a hexokinase which is specific for glucose. Hexokinase in the absence of glucose substrate is an ATPase and undergoes a conformational change (Fuente and Sols 1963). However in the presence of glucose, a larger conformational change occurs. This contraction, however, takes more time so that the rate of ATP splitting is reduced to one tenth. Lastly, if one end of the molecule is attached to a gel interface, the conformational change could provide the impetus for interface movement.

The hypothesis as expressed is still just that an hypothesis. However, in its entirely, it explains or is consistent with most of the available data on protoplasmic movement and migration in myxomycete plasmodia.

Summary

It is suggested by the author that the motility of plasmodium depends upon two mechanisms of force development. First short range forces are developed by the solation elements in the act of solation. In this process, small increases in volume occur and are important in the formation of pseudopods and advancing front areas. Superimposed upon this basic mechanism may be longer range forces developed by contractile molecules. The model implicates the metabolism in the generation of ATP which would supply the energy for the events which support the shuttle streaming with the hexose monophosphate pathway involved in the synthesis of sol.

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Theoretical consideration on movement of the myxomycete plasmodia. Dislocations and geometry of plasmodial network on cylinders and cones

The polyrhythmic protoplasmic flows in branching channels of slime mold plasmodia should be analyzed not only separately in individual channels but also geometrically through correlation and interference of waves in connected branches and anastomoses of plasmodial network.

The alternating waves of upstream and downstream reversals, accompanied by swelling and contraction respectively, travel along the channels across the plasmodial network and converge or originate in geometrically changing centres or sources (Seifriz 1952, Kamiya 1959, Stewart 1964, Jahn 1964, Jahn et al. 1964). The local expression of such changes may be the switching between the different patterns of propagation of reversal waves in connected channels. It is rather easy to sort out theoretically all possible different patterns of acceptable flows and their reversals in the simplest cases such as branching junction of three channels connected as in the letter Y, then that of two branching junctions of five channels connected as in the letter H, then of three branching junctions of seven channels and so on. After few of such steps the anastomoses connecting channels into polygonal loops must be considered.

The plasmodium moves as a complex network of channels ramifying in the above way. The changing geometrical structures of similar networks were analyzed by means of deviation from regular periodicity of crystal-like hexagonal network using the theories of the dislocations in crystals (Nabarro 1967), the topology of close-packed polygons (Cahn and Padawer 1965, Thompson d'Arcy 1942), the graphs and their coloring and flows (Berge 1962), the statistical rules of branching (Leopold 1971), etc.

The aim of the present work is the signalization of the existence of such methods and preliminary suggestions how it may be tried to interpret and measure in such terms the morphology of plasmodial networks and propagation of waves in it.

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A basic topological theorem known as Euler's Law tells us that in any closed network the corners and faces together outnumber the edges by two:

$$C-E+F=2 \tag{1}$$

or in other words the numbers of polygons with n sides times deviations from ideal hexagonal close-packing sum up to twelve:

$$\sum_{n=5,7\ldots} F_n(6-n)=12. \tag{2}$$

For example, if only small deviations from hexagons are presented then there are twelve non compensated pentagons as in geodesic domes of Buckminster Fuller, or:

$$F_5-F_7=12. \tag{3}$$

So every pentagon constitutes 1/12 of the curvature of the whole sphere and a heptagon makes a similar but negative curvature (Thompson d'Arcy 1942).

If pentagon and heptagon are neighboring polygons then the curvatures are compensated but what is left is essentially an edge dislocation (Cahn and Padawer 1965). Edge dislocation in a network of channels is the end of the extra half row of channels.

Unidirectional migration such as Anderson (1964) obtained in plasmodial network migrating on agar strips offers an interesting simplification of geometrical configurations of branching channels. Also in a plasmodium network migrating in the cylindrical surface there occurs a similar unidirectional migration but without the borders of the strip.

In such networks morphological modifications are found mainly along the direction of migration. Such a well known fact as the often occurring decrease in a number of channels from the front area towards the rear area could be represented in the simplest way by 5/7 dislocations with pentagons directed towards the front. It is shown in Fig. 1, where simplifications and distortions are not important because we consider mainly the topology of the network.

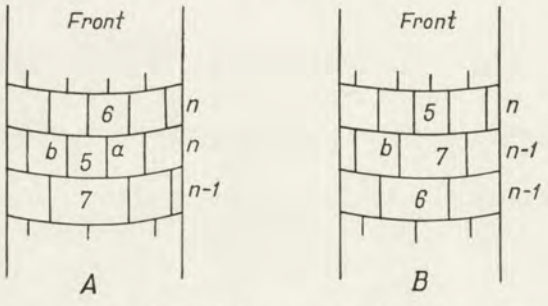

Fig. 1. Simplified representation of 5/7 dislocation climb in plasmodial network migrating unidirectionally

During migration all this moves toward the front accompanied by disappearing of some channels. It can be illustrated in Fig. 1 as reorganization of the network by shifting up the end of the extra half row of channels during which 5/7 dislocation climbs to its new 5/7 position. This is achieved by the net decrease of the diameter of channel "a" after consecutive waves of contractions and swellings, and finally retracting of this channel completely and so decreasing the number of channels from n to n–1.

At the same time this dislocation is shifted towards the right (or left if channel "b" is retracted) by a half of a complete dislocation glide. This could be regarded as the means for distribution of channels in the direction perpendicular to migration.

More complex configurational changes are also expected such as creation or anihilation of dislocations in pairs, the movement of two dislocations along different directions resulting in a third dislocation, etc. The complexity of movements and transformations of dislocations and their linear arrangements called grain boundaries (Gleiter 1971) can be appreciated best in Bragg's soap bubble model.

Topological analysis of configurations of 5/7 dislocations simplifies the problem of movements and complex transformation of dislocations and grain boundaries. It was even possible to obtain a full classification of grain boundaries and to describe their movements (Gleiter and Lissowski 1971).

The situation in the plasmodial networks of *Myxomycetes* is more complicated because of considerable heterogeneity in it when compared to almost regular lattices considered by the crystallographic theory of dislocations. Further lack of homogenity is caused in plasmodium by differences in diameters of channels. But some experience in the topological treatment of 5/7 configurations allows to look in such a way even at these complex networks.

If a plasmodial network conforms to the geometry of its position on the surface by the means of its dislocations, then the unidirectional migration on the cylinder simplifies maximally changes excluding the dislocations caused by geometrical modifications of surface. Even the closest to a uniform network on the cone or on widening strip would have to contain a 5/7 dislocations with density proportional to the slope of the cone that is to the rate of widening of available surface. Pentagons in such 5/7 dislocations are directed towards the widening.

On the cylinder there are no such "positional" dislocations and they could be smoothly included by cones of increasing slopes. The opposite of a cylinder, the case of a cone with large slope is the plane with so many "positional" dislocations that it is difficult to understand its interactions with the dislocations of a plasmodial network itself. One can vary the diameters of cylinders and cones to find out how it works with different strength of connection around them.

The complex pattern of channels and flows in a plasmodial network makes it extremely difficult to obtain quantitative results. It may be possible to obtain such results when compelling plasmodia to form such regular networks as strictly hexagonal or with given dislocations or the networks with unidirectional migration such

as on different strips, cylinders and cones. In such a network the morphological modifications and protoplasmic movements may be tried to be interpreted and measured in terms of movements and transformation of dislocations and grain boundaries.

The idea that dislocations and grain boundaries, presently the main subject of crystal physics, are also the means by which the movement of close-packed units in biology is promoted, has recently been proposed at MIT and by W. F. Harris, R. Kilkson, A. Klug, D. L. Caspar (Harris and Scriven 1970 a) for some biological assemblies of subunits such as exist in membranes, bundles of microtubules, viral sheaths, muscles, etc. This idea seems most fruitful for proper extension of recent cooperative models of membranes (Lissowski 1969, Harris and Scriven 1970 b) especially for twisting movement (slippage) elongation and bending of cylindrical surface layer in slime mold channel. It was also suggested at MIT that close-packed cells in tissue move by exchange of contacts which is essentially the dislocation glide. Even the divisions of close-packed cells occur mainly as dislocation climbs. This was achieved by considering the topological background of geometrical configurations of polygons on two-dimensional surfaces.

It is desirable for the case of a plasmodial network to work out such a theory of dislocation movements and transformations in which the elementary movement could be represented not by the traditional discrete jumping into a new configuration but by the gradual or partial redistribution of diameters of channels, during consecutive waves of twisting, supposedly also dislocated shifts in surface gel.

Maybe the simplest arrangements of flows in hexagonal networks regular or with given dislocations or in a network with unidirectional migration on strips, cylinders and cones, will indicate the centers or sources of waves. Such regions should be near the reforming channel involved in the dislocation shift.

Such changes should easier go along dislocation pathways than in other regions because of a natural requirement of minimal configurational changes similarly as in analogous conditions for the movement of dislocations and grain boundaries in crystal physics.

The preference for one direction in the networks with unidirectional migration may also answer the question (Stewart and Stewart 1959) about the direction of reversal waves: why sometimes these waves propagate towards the advancing front and in other cases they go in opposite direction.

The question of interactions of flows in parralel or serial connections of plasmodium, discussed by Stewart, Kamiya and Allen in "Primitive Motile Systems in Cell Biology" may be analyzed by detailed study of a correlation of flows at branching junction of the three channels connected as in the letter Y, then of two branching junctions of the five channels connected as in the letter H and so on.

Sometimes at branching junction of three channels the downstream (upstream) reversal wave may propagate from the strongest channel to the other two channels

as two downstream (upstream) reversal waves. But preferable for more delicate control of balanced influences between three channels is the propagation of downstream (upstream) reversal wave from the weakest channel to the strongest channel as downstream (upstream) reversal wave and afterwards as upstream (downstream) reversal wave in the third channel conforms to other waves. Preliminary observations indicate that it is often the case. The interesting patterns of flows and their reversals occur in five channels connected through two junctions as in the letter H and in six or more channels connected through triangles and other polygons. Such patterns usually repeat with some stability but sometimes one can observe switching between different possible patterns. The polyrhythmic flow is a reflection of complex interference between proximal waves from connected channels and their coincidence along the polygonal loops of channels.

Stewart (1964) has pointed out that inconstant correlation between time of reversal and time of maximal or minimal diameter at the point of the channel (Kamiya 1950) is the result of interaction between direction of channel axis and direction of waves. So this correlation also depends on the geometry of the network and such a disaccord opens the possibility for complex influences and regulation between both waves.

The statistical and structural stability of complex space time patterns of such waves, as well as that of twisting slippage – supposedly dislocational shifts propagated as controlling signals in surface gel, may compile the means for integrative control of purposeful migration of plasmodial network suggested by Stewart (1964).

The above-presented preliminary considerations will be extended and experimentally examined.

It is desirable to look for further restrictions and rules for flows in plasmodial network such as generalizations of Kirchoff rules, min-max theorems and structural stability theories of flows in networks similar to rules for coloring (Berge 1962), rules for branching hierarchies (Leopold 1971), etc. The space-time patterns of plasmodium movements have to fulfill a number of similar rules in addition to the dislocational modifications of the local contractile and elastoosmotic properties of slime mold surface layers.

Summary

Patterns of geometrical correlations of the polyrhythmic protoplasmic flows are considered preliminarily in neighboring branching junctions of connected channels of plasmodial networks migrating unidirectionally on strips, cylinders and cones and in the networks arranged regularly or with given dislocations.

The net redistributions of channels are tried to be interpreted as partial shifts of dislocations controlled by complex interference of wave patterns and their changes.

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Theo M. KONIJN

Cyclic AMP and cell aggregation in the cellular slime molds

The myxamoebae of the *Acrasieae* or cellular slime molds live in the soil where they feed on bacteria. After exhaustion of their food supply myxamoebae aggregate and form a pseudoplasmodium which migrates to the light. The cells at the tip of the pseudoplasmodium ultimately become the stalk cells of the fruiting body, while the other cells differentiate into spores.

The transition of the unicellular to the multicellular phase coincides with:

(1) the end of food uptake by the amoebae;

(2) morphological, physiological and biochemical changes in the cells;

(3) the change from an obligatory water organism to a cell mass which only differentiates in air.

One of the most intriguing questions about the life cycle of the *Acrasieae* is why the amoebae aggregate. What causes the initiation of a directed movement of the cells, which involves pseudopod formation mainly at the side of the attracting source?

Already at the turn of the century a chemotactic substance, later coined "acrasin" (Bonner 1947), was postulated to mediate the directed movement of the amoebae. Shaffer 1956, developed the first assay to test for acrasin and demonstrated that the attractant was a small, heat-stable compound that easily became inactivated in the presence of amoeba enzymes. Konijn et al. (1967, 1968), using a more quantitative assay, showed that the attractant of amoebae of the larger *Dictyostelium* species is adenosine *3'*, *5'*-monophosphate or cyclic AMP, which is secreted by bacteria (Konijn et al. 1969) and amoebae (Barkley 1969, Konijn, Chang and Bonner 1969). The importance of this nucleotide in hormone action, synaptic transmission in nervous tissues, transcription and translation of genetic information in prokaryotes, etc., has recently been reviewed in a monograph (Robison, Butcher and Sutherland 1971).

Microbiological assay for attractants of myxamoebae

Small populations of amoebae in a suspension placed on a hydrophobic agar surface stay inside the boundaries of the drop. Only at low agar concentrations (less than 0.5 per cent) do amoebae cross the boundaries of the drop on all sides.

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At an agar concentration of 0.5 per cent (Konijn 1970) the amoebae stay inside the drop on a hydrophobic surface except when they are attracted by a chemotactic source in another drop (Pl. I 1).

The attraction of amoebae by other amoebae or by bacteria can be approached in a more quantitative way by increasing the distance between the attracting and the responding drop. A response is scored positive when amoebae cross the boundary of the responding drop and move to the source of attraction. The distance over which fifty per cent of the responding drops react positively is the criterion for the activity of the chemotactic substances secreted in the attracting drops (Konijn 1970).

Another approach, which is used to test the activity of extracts or that of analogues of cyclic AMP, depends on progressive dilution of the active extract while the distance between the attracting and responding drops remains constant. In this case amoebae in the responding drop are considered to react positively if more than twice as many amoebae are pressed against the side closest to the attracting drop than on the opposite side (Pl. I 2).

Usually amoebae of *D. discoideum* are used in the responding drops. Active extracts are tested by depositing them three times at five-minute intervals to imitate the five-minute intervals between the normal pulses of acrasin secreted by aggregates.

The small population assay has been used not only to identify cyclic AMP as the attractant secreted by amoebae and bacteria, but also to test extracts of other biological materials for cyclic AMP (e.g. Van de Veerdonk and Konijn 1970). The cyclic AMP content of an extract is estimated by diluting a sample containing an unknown concentration of cyclic AMP and a solution of known concentration of commercial cyclic AMP till only about half of the responding drops react positively. Two-fold differences in cyclic AMP concentration can be shown by evoking a positive response of populations of amoebae to an active extract in a percentage similar to the percentage of response to a known concentration of cyclic AMP.

Also, the attractants of amoebae that do not react to cyclic AMP can be isolated, purified, and identified by means of this assay.

A modification of the small-population test is the slide-imprint technique. The edge of a microscope slide is pressed down on a hydrophobic agar without breaking the agar surface. The slide leaves a trough of about 0.5 mm deep. Another imprint is made with the same slide at a given angle to the first imprint, but without touching it. Amoebae are deposited in one trough and when they are in a sensitive stage tested against a cyclic AMP solution or an active extract in the other trough. The maximal distance between the two troughs over which amoebae are attracted outside their trough is a criterion for the activity of the extract.

The chemotactic activity of analogues of cyclic AMP

At the present time only two cyclic nucleotides, cyclic AMP and cyclic GMP, have been definitely shown to occur naturally (see Robison et al. 1971). Cyclic AMP

is more active than cyclic GMP in most biological systems (see Jost and Rickenberg 1971). N^6-*2'-0*-dibutyryl-adenosine-*3'*,*5'*-monophosphate is the most commonly used synthetic analogue of cyclic AMP. Normally it is more active than cyclic AMP, supposedly because it penetrates into the cell more easily and is not broken down by adenosine *3'*,*5'*-monophosphate phosphodiesterase (see Robison et al. 1971).

Generally cyclic AMP acts intracellularly. To test its mode of action cells are often homogenized. If studied in vivo, extracellularly applied cyclic AMP does not penetrate the cell membranes of several tissues. Even if it passes the membrane one does not know how many molecules enter the cell, and at which rate.

The amoebae of the cellular slime molds offer several advantages for use as test organisms for cyclic AMP and its analogues. They react to extracellularly applied cyclic nucleotides. Cyclic AMP is active at the outer cell membrane and does not have to penetrate into the cell, at least not at appreciable levels, to induce a chemotactic movement (Peter B. Moens, personal communication). Apparently cyclic AMP stimulates the amoeba membrane in a way which is similar to the activation of the

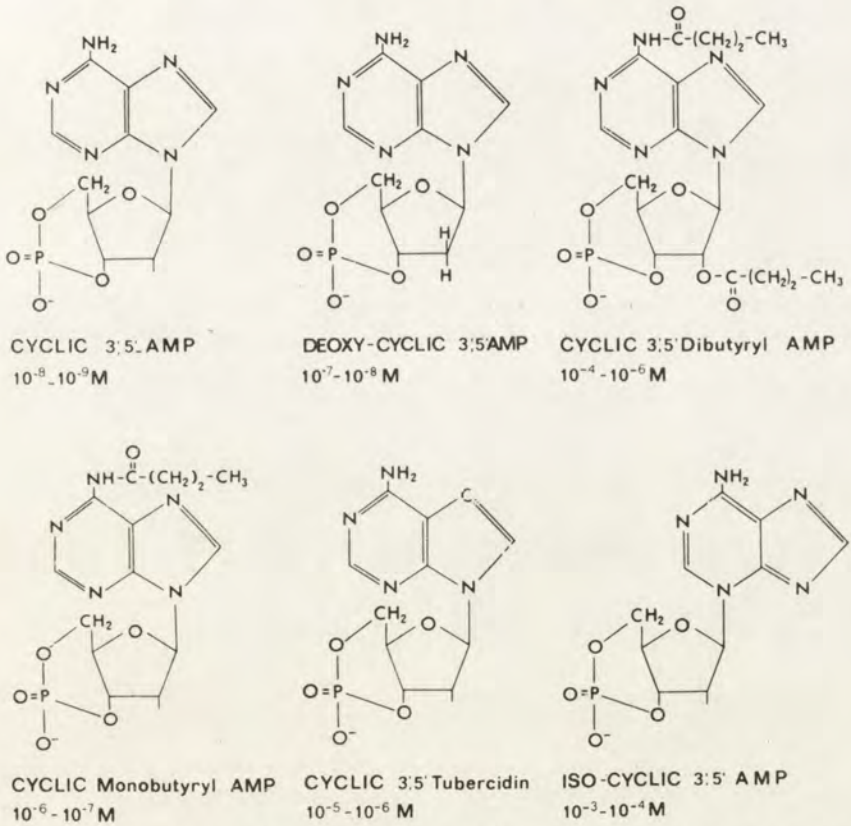


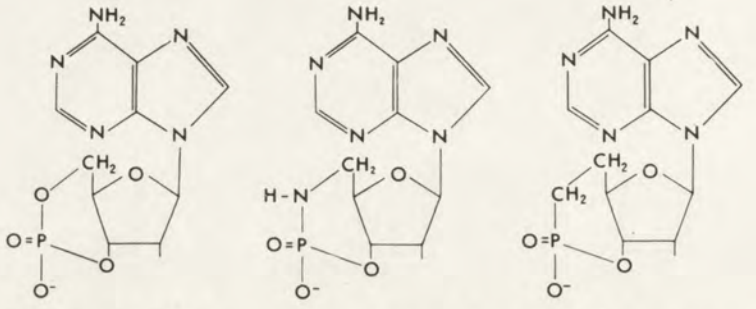
Fig. 1. Chemotactic activities of analogues of cyclic AMP. Fifty percent of the populations react positively between the given molarities

membranes of target cells by hormones. Myxamoebae are free-living cells and do not have to be disaggregated, as is required to obtain cell cultures of tissues of multicellular organisms.

The assay described in the previous section has been used to test the activity of various cyclic nucleotides and their analogues.

The order of the chemotactic activity of cyclic nucleotides as tested with amoebae of *D. discoideum* is: cyclic AMP > cyclic UMP > cyclic CMP ≥ cyclic GMP > cyclic IMP ≥ cyclic TMP ≥ cyclic XMP (Konijn 1972). The myxamoebae are not preferentially attracted by cyclic nucleotides containing a purine base. Slight changes in the base moiety, as in tubercidin *3',5'*-monophosphate (Fig. 1), may result in a strong reduction of chemotactic activity.

Changes in the ribose moiety, and in the configurational binding of the ribose moiety to the base moiety greatly affect chemotactic activity (Fig. 1). The attraction of myxamoebae by commercial dibutyryl cyclic AMP may be increased by the presence



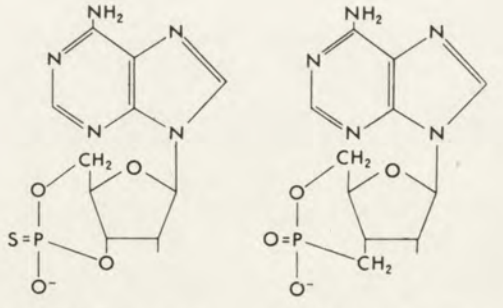


Fig. 2. Chemotactic activities of analogues of cyclic AMP with substitutions in the phosphate moiety

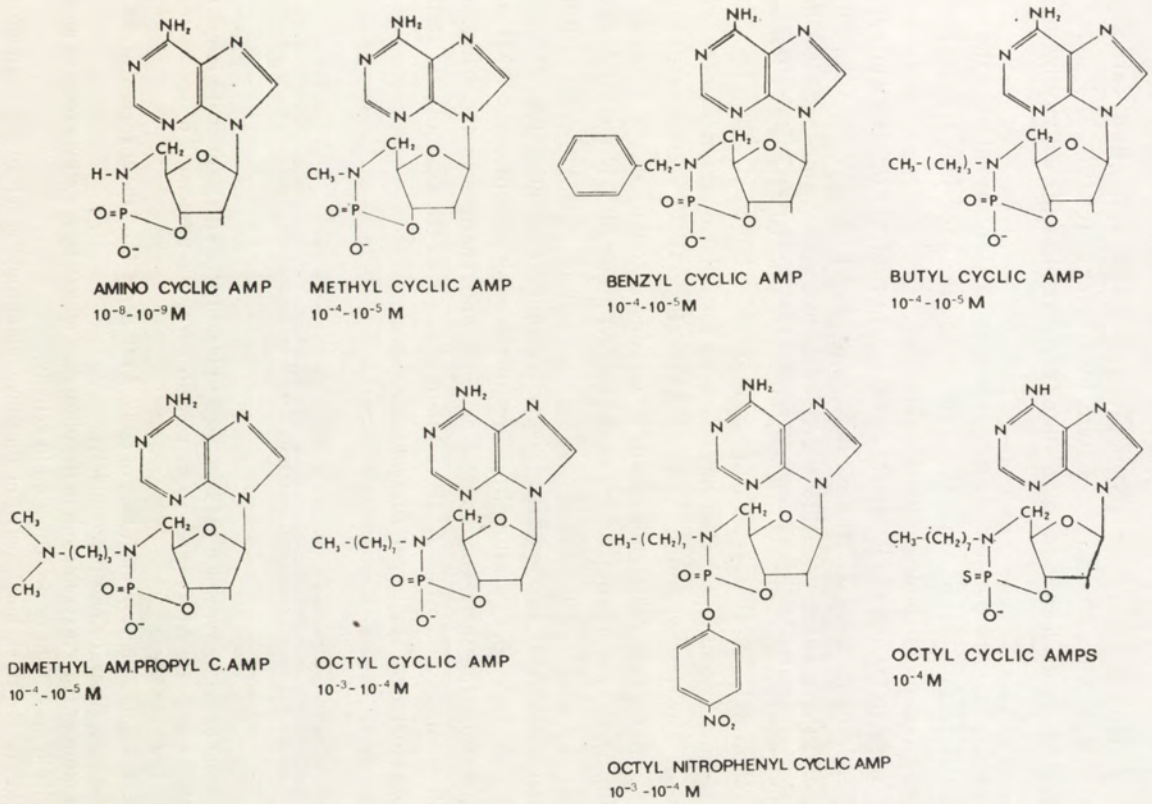


Fig. 3. Chemotactic activities of 5′-amido analogues of cyclic AMP

of traces of monobutyryl cyclic AMP and cyclic AMP itself (Konijn 1972). In deoxy-cyclic AMP the decrease of activity is only slight in comparison with the reduction in activity if other changes are introduced in the molecule.

The most essential part of cyclic AMP is the phosphate moiety. Changes in this part, such as replacement of the oxygen atom connected to the third carbon atom of the ribose, results in a reduction of activity by a factor of 10^6 (Fig. 2). Replacement of the double-bond oxygen atom by a sulfur atom reduces the activity by a factor of 10^2.

No, or only a slight, reduction in chemotactic activity is observed when the oxygen atom between the fifth carbon atom of the ribose and the phosphate atom is replaced by a methyl group or an amido group (Fig. 2).

I am grateful to Dr. Bernd Jastorff (Max-Planck-Institut, Göttingen), who supplied several 5′-amido analogues which have been tested in various systems for their biological activity (Murayama et al. 1970). All analogues attract amoebae of *D. discoideum*. 5′-N-methyl-5′-desoxy-cyclic AMP, however, was 10^4–10^5 times less active than 5′-amino-cyclic AMP (Fig. 3). Substitution of the methyl group by a butyl, octyl, or a dimethylaminopropyl group results in a similar or slightly lower activity than that of 5′-N-methyl-5′-desoxy-cyclic AMP (Fig. 3). Oxygen substitution by sulfur in the phosphate moiety also leads to a reduced activity of the 5′-amido analogues (Fig. 3). A benzyl group added to the methyl group of 5′-N-methyl-5′-desoxy-cyclic AMP does not change the chemotactic activity. 5′-N-octyl-5′-desoxy-p-nitrophenyl-ester-cyclic AMP is one of the least active amido analogues (Fig. 3).

In summary, the configurational features of the analogues of cyclic AMP are of great importance for their chemotactic activity, and changes in the base, sugar, or phosphate moiety of cyclic AMP result in changes in the chemotactic response of the myxamoebae of the larger *Dictyostelium* species.

Summary

A microbiological assay, which has been used to identify the attractant of the larger *Dictyostelium* species as cyclic AMP, can also be used to purify the attractants of species that are insensitive to cyclic AMP.

The chemotactic activity of analogues of cyclic AMP depends on the changes that are introduced in the base, sugar, or phosphate moiety.

Substitution of the 5′-oxygen atom in the phosphate moiety by a methyl group or an amido group results in very slight or no reduction in activity.

Addition of a methyl, butyl, octyl, or dimethylaminopropyl group to the 5′-amido group in the phosphate moiety reduces the chemotactic activity by a factor of 10^4–10^6.

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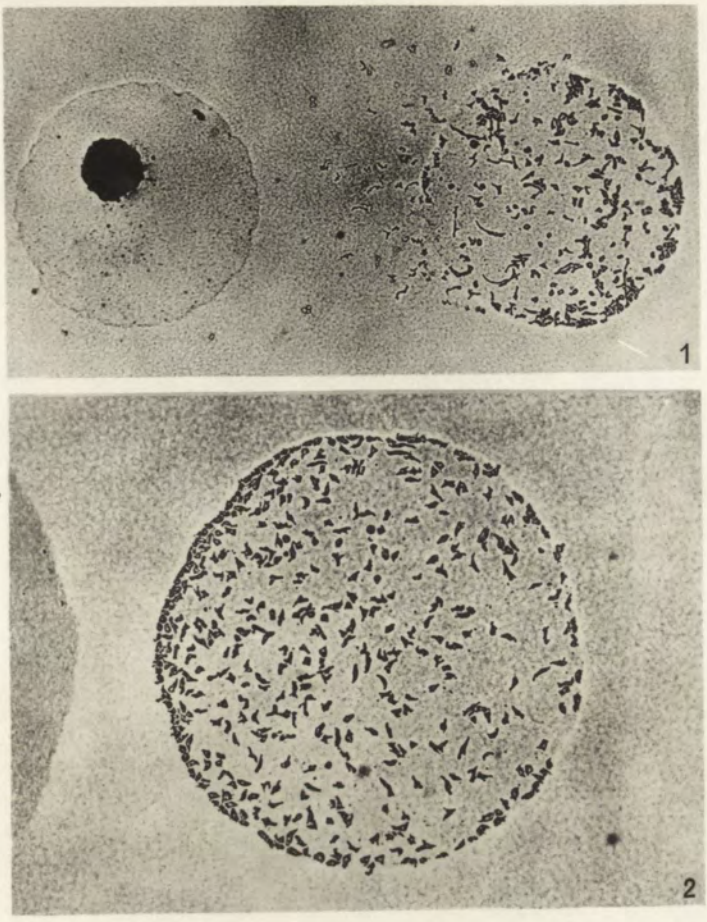
EXPLANATIONS OF PLATE I

1: Chemotaxis in *Dictyostelium discoideum*. The aggregation in the drop on the left attractcts myxamoebae outside the boundary of the drop on the right (From Konijn, Experiemtiaia, 1970) ×60

2: Drops (0.1 µl) containing 3×10^{-13} g cyclic AMP were deposited three times at 5 min imterervals to the left of the responding drop (From Konijn, Experientia 1970) ×95



T. M. Konijn auctor phot.

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Surface movement in fibroblast locomotion

When placed in tissue culture on a glass substratum, virtually all cell types of higher animals will undergo some locomotion. While the very considerable diversity of cell morphology may be reflected by some apparent differences in cell locomotion, it appears that most such cells move by fundamentally the same mechanism. With the possible exception of leucocyte locomotion, tissue culture cell movement differs greatly from that of amoebae as usually conceived. There is no directional cytoplasmic flow, but instead locomotion is associated with an active upfolding movement of the cell's leading edge, which is called "ruffling" (Abercrombie 1961).

Such cellular movement is presumably important in wound healing, embryonic development, and probably also cancer. Cell locomotion is, however, most conveniently studied in tissue culture, where the cellss pread upon a plane transparent substratum, usually glass, where their movements can be analysed in the minutest detail using phase contrast microscopy. Although it can be truly said that such an environment and substratum are abnormal, nevertheless cells do move both in the body and in culture, and it seems improbable that they would possess separate mechanisms for each type of movement. Furthermore, although Ross Harrison originally developed the tissue culture method specifically to study cell locomotion, the embarrassing fact is that the mechanism of this locomotion has yet to be discovered.

This paper and its accompanying film are principally concerned with the results of marking the cell surface with small particles. It reviews work done by Abercrombie et al. (1970 a, b) Ingram (1969), and myself (Harris 1971), as well as presenting new and as yet unpublished observations made in collaboration with Michael Abercrombie, Graham Dunn, and Jennifer Allen, of the Strangeways Research Laboratory.

Methods

The phenomenon of marker particle movement has now been observed on a variety of cell types. These include chick heart fibroblasts (Abercrombie et al. 1970 b, Harris 1971), 3T3 line mouse fibroblasts (Harris 1971), BHK hamster fibroblasts (Harris 1971), S-180 mouse sarcoma

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cells (Harris 1971) and KB line human carcinoma cells (Harris 1971). Most recently a very similar phenomenon has been encountered in mouse peritoneal fibroblasts (Harris, Allen unpublished). Indeed no cell types have been encountered in which particle transport differs substantially from that which is to be reported here. Likewise all types of particles which have been used as markers have been found to be transported in exactly the same ways, so long as they adhere to the cell surface. Neither does the size of particle seem to matter, at least over a range of approximately 0.25 to 5 μ diameter. The types of particle which have been used are India ink (Abercrombie et al. 1970 b, Harris 1971, Ingram 1969), ground charcoal (Harris 1971), molybdenum disulfide (Abercrombie et al. 1970 b), ground anion exchange resin (Harris 1971), ground cation exchange resin (Harris 1971), colloidal gold (Harris 1971), and powdered nickel (Harris et al. unpubl.).

The film presented shows the movement of ground anion exchange resin particles on chick heart fibroblasts and upon cells of the 3T3 line of mouse fibroblasts. These cells were cultured in Dulbecco-Vogt modified Eagle's medium supplemented with 10% fetal calf serum, and adjusted to pH 7.2 with HEPES buffer. Other standard media have been used in similar studies without altering the pattern of particle movement in any apparent way.

Films were taken using both Arriflex and Wild time lapse equipment on Plus-X Reversal film. The interval between exposures was 10 sec, except in the initial sequence, in which it was 30 sec. Zeiss phase contrast (Zernike) equipment has been used throughout.

Background and observations

The fully spread cells have a rather thickened central region, containing the nucleus and the greater number of mitochondria and other organelles. From this extend one or more, usually several, broad flattened extensions which have beeen called ruffled membranes, major processes, or even pseudopodia. Following Ingram (1969) and Abercrombie (1970 a), I shall refer to these extensions by the more noncommittal term of "lamellae". In a healthy moving cell, the outer margin of these lamellae undergoes the process known as ruffling. Ruffling consists of the repeated formation of thickened areas at the cell margin and their movement a short distance inward before being resorbed. As Ingram (1969) and I (Harris 1969) have shown, this ruffling consists of repeated upfolding of the outermost part of the lamella and its gradual bending inward. Ruffling is closely correlated with locomotion; cells rarely move for any distance without ruffling, and those parts of the margin which ruffle most actively typically undergo the most spreading. Furthermore it has been shown that contact between many cells results in a localized cessation of ruffling at the point of contact as well as inhibiting further outward movement at the point of contact (Abercrombie and Ambrose 1958, Trinkaus et al. 1971). Thus it is believed that ruffle inhibition is closely related to contact inhibition of locomotion as described by Abercrombie (1970).

Another important type of surface movement is blebbing (Price 1967). This consists of the rapid protrusion of hemispherical protuberances from the cell surface, apparently by hydrostatic pressure (Harris 1971). Although blebbing often occurs over the entire cell surface, as for example in detached cells, it is very often localized

at the lamellar margin where it may alternate with ruffling. Such marginal blebbing is also inhibited by intercellular contact (Harris 1971).

When small particles are sprinkled onto the cell surface, or are placed on the substratum in the path of an advancing cell, these particles will be actively transported centripetally on the cell surface. This movement is relatively steady, although there may be momentary pauses and changes in direction. Particles move from areas of ruffling or blebbing (usually an advancing lamella) toward the central nuclear region of the cell. The usual rate of movement is from 1 to 6 μ/min, generally about 2 μ/min (Abercrombie et al. 1970 b). Particles accumulate in the vicinity of the nucleus, either overlying it or stopping just before reaching the nucleus itself and collecting in a ring around it. Particles often show a similar disinclination to move directly over large vacuoles and will often detour around them or collect in a ring around them. Less frequently, particles also collect in discrete patches on the cell surface at some distance from the nucleus. Such patches are commonly found at the sharply concave parts of the cell margin between the lamellae. The same pattern of movement is observed whether the cells are grown substrate down or substrate up (hanging) so the disinclination of particles to move above large inclusions is presumably not due to gravity or the cell's surface profile per se (Harris 1971).

Cultured fibroblasts do sometimes phagocytize particles, and such particles move in much the same way, so it is important to distinguish with certainty movement upon the cell surface from movement within the cell. The particle movement described above definitely occurs on the outside surface of the cell, as has now been determined by a variety of methods. First, high numerical aperture objectives have such extremely thin depths of field that it is possible to determine the vertical position of small particles within one or two microns. By careful vertical focusing it can be shown that at least the larger marker particles (above 2 μ diameter) lie outside the cell during movement. By swelling cells with hypotonic medium (below 1/10 normal tonicity) it is possible to raise the cell membrane far above the previous level of the cell surface while leaving most cytoplasmic inclusions in their previous positions. By this means it has been found that the great majority of collected particles remain outside the cell membrane. It has also been possible to micromanipulate the attached particles and move them about on the cell surface or even detach them. Furthermore, in especially elongate cells it is frequently possible to gain a side view of the attached particles, revealing their external position, and such particles may even fall off the cell surface. Abercrombie et al. (1970 b) have published a vertical electron micrograph section through a fibroblast transporting a particle, and this particle can be seen to lie entirely outside the cell membrane.

A variable percentage of the particles encountered by cells are not picked up and transported, but instead remain attached to the substratum while the cell moves over them. Under some conditions particles are picked up and transported centripetally on the lower (substratum) side of the cell. Such particles move steadily inward at the usual speed but then stop about 4–6 μ inward from the margin as though wedged

between cell and substratum. The cell then moves over them, and they undergo little additional movement except for occasional quivering. This phenomenon has so far been observed with chick heart fibroblasts only, and only when they are cultured in HEPES buffer (Harris et al. unpubl.).

Attention has also been paid to the relative movement of nearby particles. In most cases this movement is highly correlated, particles moving in approximately parallel or somewhat convergent paths, at very nearly the same rate. It is particularly to be noted that momentary pauses or changes in direction occur simultaneously in nearby particles, indicating that the surface is itself moving as a sheet. A particularly dramatic sequence is included in the film, in which two particles approximately 4 μ apart trace out complicated but nearly identical paths across the cell surface, changing direction and speed synchronously. This is taken as indirect evidence that the cell membrane is itself moving and that particle movement is a reflection of this (Harris et al. unpubl.).

Discussion

Because of the consistency of particle movement observed on such a variety of cells, it seems likely that any movable particles adhering to the upper surface of any ruffling, blebbing, or spreading cell can be expected to be transported centripetally and/or in a direction opposite to that of cell locomotion. Indeed even retraction fibers, thin flexible extensions of neighboring cells, are transported in this way when adhering to the upper cell surface. It also seems probable that this particle transport represents a sort of mirror image consequence or byproduct of the (still unknown) locomotory mechanism itself. On the lower surface the cell pulls centripetally against the immovable substratum, thus pulling itself outward. On the upper surface this tractional force would be applied to movable particles, which would be conveyed centripetally.

Abercrombie et al. (1970 b) have made the plausible suggestion that the membrane itself moves continuously in a centripetal direction on both upper and lower surfaces, propelling the cell and carrying the particles with it. They have also hypothesized that new membrane is continuously being assembled at the leading margin and that the force of this assembly is what propels the cell.

Alternatively it has been suggested (Harris 1971) that membrane flow in tissue culture cells may be a byproduct of locomotion, the membrane being pulled centripetally by some cytoplasmic components, probably microfilaments. Besides causing the membrane to flow, this tension would tend to shift the equilibrium between assembly and disassembly, thus localizing assembly in areas of greatest stress. Both of these hypotheses are of course quite similar to that proposed by Shaffer (1962) to explain the locomotion of *Dictyostelium* and the movement of marker particles on the surface of that organism.

If we postulate that each small portion of membrane remains stationary with

respect to whatever it is adhering to, whether particle or substratum, then, on the basis of current evidence, not only must the membrane flow centripetally, but additional membrane material must continually be added at the margin. This new mambrane might reach the margin either on the surface or through the cytoplasm. The former seems improbable since all particles are conveyed centripetally on either the upper or lower cell surface and none move outward. It is not impossible that only unattached or non-adherent parts of the membrane flow outward, as for example might be true if adhesion to particles actually induced bits of the membrane to move inward. This however seems unlikely since particles of all sizes are moved in the same fashion, as would presumably not be the case if the particles themselves were inducing membrane flow. Also, the highly correlated movement of adjacent particles indicates that movement occurs as a single sheet rather than in isolated patches.

It has been suggested that membrane material might reach the margin in the form of small vesicles within the cytoplasm. However, while vesicles are often observed within the lamellae, these are pinocytotic vacuoles which are formed at the margin and move inward. No net movement of any vesicles has been observed from center to margin. Thus it appears that, at the lamellar tip, new membrane is continually being assembled from invisibly small precursors or subunits (less than 0.2 μ in diameter).

This assembly must occur at a rate commensurate with that of particle movement and cell locomotion. For example, a cell spreading outward at a rate of 1 μ/min while transporting particles inward at a rate of 2 μ/min (movement relative to substratum) would assemble at least 4 μ^2 of new membrane per μ of leading edge (rate of particle transport + twice rate of locomotion). For such a cell to move a distance equal to its own length (which might take 2 hours or less), it would have to assemble and reassemble an amount of membrane equal to twice its surface area.

This is vastly higher than the reported rate of turnover of membrane constituents as determined by radioactive incorporation studies (Warren and Glick 1968), and so this turnover must represent re-use of undegraded materials. Nevertheless such a high rate of assembly and flow would be expected to betray itself in some of the many studies which have been made on membrane constituents. In fact, the theory of flow set forth above can account for a number of previously unexplained phenomena encountered by workers in this field.

For example, Marcus (1962), studing the appearance of myxovirus-produced hemagglutinin in the cell membrane of newly infected cells, found that this protein appears first at the margin, especially near ruffling, and moves gradually inward. H. Harris (1970), observing the location of surface antigens upon fused cells of different types, has found that these antigens become rapidly redistributed and mixed soon after fusion. Furthermore, when new surface antigens begin to be synthesized, these appear first at the lamellar tips from which they move inward.

Because ruffles and blebs occur most frequently at the lamellar tip, and because particles are transported away from areas of such activity even when it occurs at non-

marginal parts of the cell surface, it would appear that both types of activity represent rapid localized assembly of new membrane. In fact there are additional reasons for believing that blebbing involves such assembly. It has been shown that rapid perfusion of cells with strongly hypertonic medium causes blebs to shrivel rather than simply to retract, as would have been the case if the expansion were simply due to stretching (Harris 1971). Most recently we have found that even when cells are entirely coated with attached particles, newly produced blebs are entirely clean of these particles (Harris et al. unpubl.). Both observations tend to confirm that blebs consist of newly assembled membrane rather than either stretched membrane or membrane derived directly from surrounding areas. By extension, contact inhibition of ruffling, blebbing, or locomotion may represent inhibition of membrane assembly. Since intercellular contact inhibits not only ruffling and blebbing, but also particle transport, it would appear that such contact somehow prevents membrane assembly. If so, the much studied phenomenon of contact inhibition would amount to the prevention of membrane assembly at points of intercellular adhesion. Conceivably the two opposed membranes could simply reinforce each other. Work on this problem is continuing.

Summary

Vertebrate tissue culture cells of all types studied transport any of a variety of particles adhering to their surface. The particles are transported centripetally, in the direction opposite that of cell spreading or locomotion. It is believed that this particle movement represents the continuous net inward flow of the surface membrane and that this is closely related to the mechanism by which the cell exerts traction on solid substrata. Such net membrane flow necessarily entails a high rate of membrane assembly at the cell margins, and the implications of this assembly are discussed.

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A possible role for actomyosin-like protein in cell adhesion and motility

One of the interesting facts that has arisen from studies on actomyosin-like proteins in non-muscle tissues and cell systems (see review by Poglazov 1966) is that these proteins with their rudimentary contractile properties are utilized for many different purposes. The function of the protein system depends on the mechanism with which it is associated.

It was therefore to be expected that the finding of a myosin-like protein in the limiting membrane of adult rat liver cells (Neifakh and Vassiletz 1965, Neifakh, Avramov, Gaitskhok, Kazakhova, Monakhov, Repin, Turovski and Vassiletz 1965, Vassiletz and Zubzhitski 1966) invited speculation about the function of this surface-localized protein. Poglazov (1966) put forward the suggestion that it might be connected with permeability, but there is no evidence to support this belief. An evaluation and comparison between cell aggregation experiments with nucleotides, ethylenediaminetetra-acetic acid (EDTA), ouabain and mersalyl (Salyrgan) and the properties of actomyosin pointed to the possibility of this surface-localized protein with its (Ca^{++}, Mg^{++})-dependent ATPase activity being involved in some manner with cellular adhesiveness (Jones 1966, 1967).

We might consider first the experimental data upon which this hypothesis was originally based. To begin with the presence of a myosin ATPase at the cell surface was consistent with there being considerable ATPase activity at the surfaces of cells. This was borne out by the fact that exogenous ATP was rapidly split in the presence of platelets (Salzman, Chambers and Neri 1966) and of dissociated embryonic chick cells (Knight, Jones and Jones 1966). And Salzman et al. (1966) went as far as to propose that the platelet "ecto-ATPase" responsible for splitting the exogenous ATP was thrombosthenin, the (Ca^{++}, Mg^{++})-dependent myosin ATPase of platelets (Bettex-Galland and Luscher 1965). However, the possibility of a (Na^{++}, K^{+}, Mg^{++})-dependent ATPase being engaged in this process could not obviously be ruled out.

It was also of interest that the activity of myosin ATPase (Bowen and Kerwin 1954) and the ability of cells to aggregate (Pl I 1) were completely abolished in the

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presence of ethylenediaminetetra-acetic acid (EDTA), which binds calcium and magnesium ions. Was it too far-fetched to assume that part of the answer to why cells failed to adhere to one another in the absence of these ions in the ambient medium might lie in an accompanying inactivation of the surface-localized (Ca^{++}, Mg^{++})-dependent myosin ATPase? There was certainly room for this assumption because the role of calcium in cell adhesion has been a subject of much controversy ever since Herbst demonstrated its importance in his cell reaggregation studies (1900).

In connection with cell adhesion it has been cogently argued that calcium by cross-linking negative sites reduces the electronegative forces of repulsion sufficiently for the cells to overcome the electronegative barrier so that they can approach close enough (5–10 Å) for the calcium to form intercellular bridges (Pethica 1961). Curtis (1966) preferred to believe that calcium performed this function of reducing the repulsive forces in so far that it brought cells to within a distance of 150Å–200Å. At this separation the cells, it was suggested, were held in position by the electrostatic forces of repulsion and attraction being balanced in accordance with the tenet of the proposed physical forces of cell adhesion (Curtis 1960). However the evidence in support of this theory is slight (Weiss 1968) and the results of electrophoretic mobility studies do not support it (Weiss 1968, Kemp and Jones 1970). The suggestion by Pethica (1961) that cells approach close enough to form intercellular adhesions and that calcium aids this process by reducing the repulsive forces therefore seems to be nearer to the true situation.

It is likely that the role of calcium in adhesion is many-sided. If cell adhesion were to some degree dependent on the activity of the (Ca^{++}, Mg^{++})-dependent surface-localized myosin ATPase and on the energy harnessed by this protein system, then the inability of cells to aggregate in the presence of EDTA (Pl. I 1) could be partly explained in terms of the myosin ATPase being inactivated by the depletion of calcium and magnesium ions.

In keeping with this assumption that cell aggregation may be affected by altering the activity of the surface-localized myosin ATPase were the results of aggregation experiments with exogenous nucleotides. When ADP was introduced into platelet rich plasma, it was found that the otherwise non-adhesive platelets aggregated (Gaarder, Jonsen, Laland, Hellem and Owren 1961). Also, in the presence of either ADP or ATP the aggregative competence of rotated embryonic chick cells was markedly reduced (Knight et al. 1966, Jones and Kemp 1970). A reasonable explanation for these effects was that ADP, through product inhibition (Kielley and Kielley 1953) blocked the activity of the surface ATPase systems (Jones 1966, Salzman et al. 1966). It will already be apparent, however, that one of the shortcomings of these experiments with nucleotides in relation to surface-localized myosin ATPase being connected with adhesion was that the sodium pump ATPase could equally be put out of action by ADP.

Although constraint over interpreting results obtained by use of chemical inhibitory agents is desirable, the ability of mersalyl (Salyrgan), the most specific inhibitor

of myosin ATPase activity available (Bettex-Galland and Luscher 1965), to reduce considerably the aggregative competence of embryonic chick cells without affecting viability seemed significant (Pl. I 2). The failure of ouabain, which specifically blocks the activity of the sodium pump ATPase, to produce the same effect, indicated that the surface myosin ATPase was more closely involved than the sodium pump ATPase with cell adhesion.

When the data yielded by these experiments were collected together they seemed to point to the possibility of the myosin-like protein being connected with the adhesive mechanism. This possibility was tentatively put forward to serve as a working hypothesis (Jones 1966, 1967).

We might now turn to the recent immunological experiments that were designed to test this hypothesis. The aim was to make the surface myosin-like protein the exclusive target of anti-myosin antibodies. If this could be achieved, then, providing the hypothesis was valid, one would expect the inactivation of the myosin ATPase by specific anti-myosin antibodies to influence the aggregative competence of the test cell system. This approach seemed to be justified when Chambers, Salzman and Neri (1967) reported that rabbit antisera to platelet actomyosin (thrombosthenin) induced platelets to aggregate and simultaneously blocked the ATPase activity of this protein. Unfortunately, the reference to these findings was exceedingly brief and accordingly drew forth the criticism that the antiserum may have been acting as an agglutinant and that contaminant antibodies may have been responsible for the platelet aggregation effect.

In view of this legitimate criticism it seemed preferable to use cells that normally aggregated readily, and to determine whether or not anti-myosin antibodies directed against the surface-localized myosin-like protein significantly reduced the aggregative competence of these same cells. It was also necessary to obtain an anti-myosin antibody preparation in which anti-transport ATPase, anti-tropomyosin, anti-collagen and anti-glycoprotein antibodies were virtually absent.

Trypsin-dissociated embryonic chick cells since they aggregated readily on rotation provided a suitable test cell system. Judging from the finding that both the ATPase activity and actin-binding capacity of myosin were retained after 10–12 min treatment with trypsin (Mihalyi and Szent-Györgyi 1953), it seemed unlikely that the brief treatment of these cells with 0.25% trypsin would impair the activity of myosin at the surface. However in this connection it was essential to determine whether a myosin-like protein was present at the periphery of these cells. The technical difficulties of extracting and purifying such a protein from the membranes of these cells and preparing antibodies against it were insurmountable. In our joint investigations, Dr. Gröschel-Stewart, Dr. Kemp and myself realized that the problem of detecting whether or not myosin-like protein was present at the surface of embryonic chick cells could be overcome because rabbit antisera prepared against purified smooth muscle actomyosin from human uterus (anti-UAM) or chick gizzard (anti-GAM) was found to react, as evidenced by use of the actomyosin-fluorescent antibody technique, with

the surfaces of non-muscle cells, for example fibrocytes and platelets (Gröschel-Stewart, Jones and Kemp 1970; Jones, Kemp and Gröschel-Stewart 1970). Antibodies prepared against striated pectoral muscle actomyosin (anti-PAM) failed to react with non-muscle cells. The indication was that the immune properties of smooth muscle actomyosin and of myosin-like protein at the surfaces of non-muscle cells were similar but different from those of striated muscle actomyosin. An important finding was that the target of the antibodies against both the smooth and striated muscle actomyosin was the myosin ATPase; these same antibodies did not react with the (Na^+, K^+, Mg^{++})-dependent transport ATPase of human erythrocytes (Gröschel-Stewart 1969).

The anti-chicken smooth muscle actomyosin antisera (anti-GAM) did not form a precipitin band with tropomyosin. The presence of anti-collagen and anti-glycoprotein antibodies in the anti-chicken and anti-human sera is also improbable because preliminary immunodiffusion studies have shown that ground substance and anti-human smooth muscle actomyosin sera or the reciprocal cross do not react (Kemp, Jones and Gröschel-Stewart 1971). The results of the interactions between anti-chicken smooth muscle actomyosin fluoresced antibodies and embryonic chick muscle and liver cells previously reported by Gröschel-Stewart, Jones and Kemp (1970) are shown in Plate II 4,5. Anti-striated muscle actomyosin antibodies (anti-PAM) and non-immunized rabbit serum (NIS) did not react with these same cells (Pl. II 6, 7). It was concluded from these experiments that a myosin ATPase system was present at the surfaces of embryonic chick muscle and liver cells.

We have also shown that anti-human smooth muscle actomyosin (anti-UAM) and anti-chicken smooth muscle actomyosin antibodies (anti-GAM), which are capable of specifically reacting with the surface-localized myosin-ATPase of embryonic chick muscle and liver cells, markedly inhibited the aggregative competence of these same cells (Jones, Kemp and Gröschel-Stewart 1970, Kemp, Jones and Gröschel-Stewart 1971). Anti-striated muscle actomyosin sera (anti-PAM), non-immunized rabbit sera (NIS), and calf serum did not produce this aggregation-inhibitory effect. The results of these aggregation experiments are shown in Plate III, 8–11. The immunological experiments thus strengthened the evidence for surface-localized myosin ATPase being involved in cellular adhesion (Jones 1966).

Mr. Evans, Dr. Kemp and myself have recently found that the aggregative competence of embryonic chick cells is reduced after treatment with a preparation of monovalent fragments of Concanavalin A (Con A) obtained by using trypsinization and fractionation procedures. Since Con A reacts specifically with carbohydrates the indication is that the carbohydrate part of a glycoprotein (cell-binding component) seems to carry the adhesive site. Bretscher (1971) has produced evidence in support of the carbohydrate part of one of the major glycoproteins at the periphery of the red cell being placed upon the exterior surface of the membrane. Here the carbohydrate is attached to the polypeptide chain which thereafter alone extends through

the membrane to lie below the lipid bilayer. Bretscher (1971) suggests that glycoproteins at the periphery of most cells are likely to be similarly arranged. So presumably could a surface-localized myosin molecule.

These findings are in keeping with the view (Jones 1966, 1967) that conformational changes in a surface-localized myosin-like contractile protein might induce similar changes in an adjacent glycoprotein which carries the binding site. Accompanying changes in the binding site could render it favourable or unfavourable to adhesion.

Whatever the nature of the adhesive mechanism it is evidently responsible for at least three distinguishable kinds of adhesions.

(1) Adhesions that subserve cell motility in the sense that they are utilized by the cell to gain purchase in order to move.

(2) Non-selective adhesions that occur between cells of different type in mixed aggregates; these adhesions are relatively weak and allow cell separation which can lead, in turn, to the cells moving relative to one another (Jones and Morrison 1969).

(3) Selective adhesions which are more permanent and are established between cells of the same type in mixed aggregates (Steinberg 1964). The differences between these types of adhesions can be translated into differences of strength relative to motility. Differences in adhesiveness between cells of different type come into another category and the answer to this particular problem must lie in the binding sites and the bonds they form (Steinberg 1964, Jones and Morrison 1969).

Despite being closely bound up with one another there is every reason to believe that adhesion and motility are phenomena which are served by separate mechanisms. The presence of an actin-like protein system in the cortical region of cells (Ishikawa, Bischoff and Holtzer 1969) may not constitute proof that it is connected with motility. This system is nevertheless suitably placed to set in motion the undulating movements that are associated with locomotion (Ambrose and Forrester 1968). The crests of the undulating surface of a cell make the contacts required for cell movement.

The fact that cells display a great variety of movements has not been overlooked, and in assigning to the actin-like protein system in the cortical region a possible role in locomotion, it does not imply that a single mechanism is responsible for the varied movements. For the protein system in question to perform the function proposed there would be a need for other mechanisms and other requirements. The protein system could not do work unless for example the endoplasmic region of the cell were used as a kind of "hydrostatic skeleton" and the system was attached at intervals to the membrane.

The accumulated evidence suggests that the adhesive mechanism is located at the cell surface. The proposed model suggests that the surface-localized myosin-like protein is exclusively concerned with modulating the behaviour of cell-binding components in its immediate vicinity, and, as a result of this, changes in the disposition of the binding sites.

For undulating movements of a cell surface or conformational changes in surface-localized cell-binding components to come within the competence of a mechanism, a requirement, it seems to me, is a driving system with a capacity to harness energy. An actomyosin system is designed for this purpose. It is strategically well placed in cells to play such a role and the data available at the present time seems to point to actomyosin-like protein being associated with both cell adhesion and motility.

Summary

Cell aggregation experiments with nucleotides, EDTA, mersalyl and in particular with anti-actomyosin antibodies capable of reacting with surface-localized myosin ATPase point to this protein system being involved in cell adhesion. It is suggested that the actin-like protein system in the cortical region of cells provides the motive force for the surface undulating movements associated with cell motility. If cell adhesion or motility are to come within the competence of a mechanism, this is likely to be controlled by a driving system with a capacity to harness energy. An actomyosin system is uniquely designed to perform this function and the accumulated evidence seems to suggest that this protein system has a role in the regulation of cell adhesion and motility.

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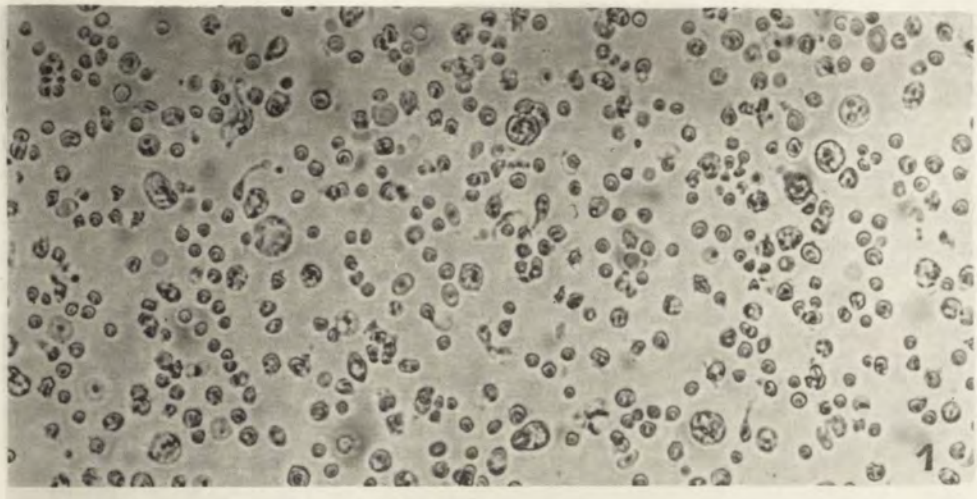
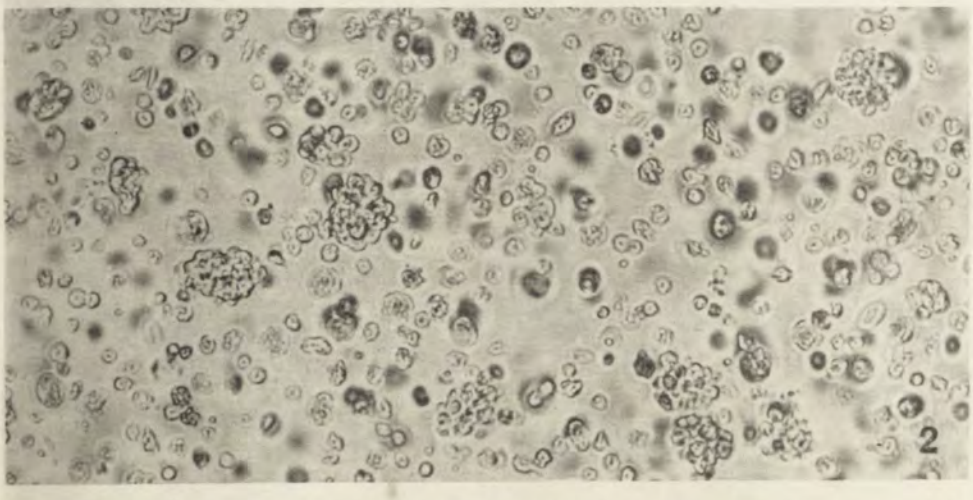
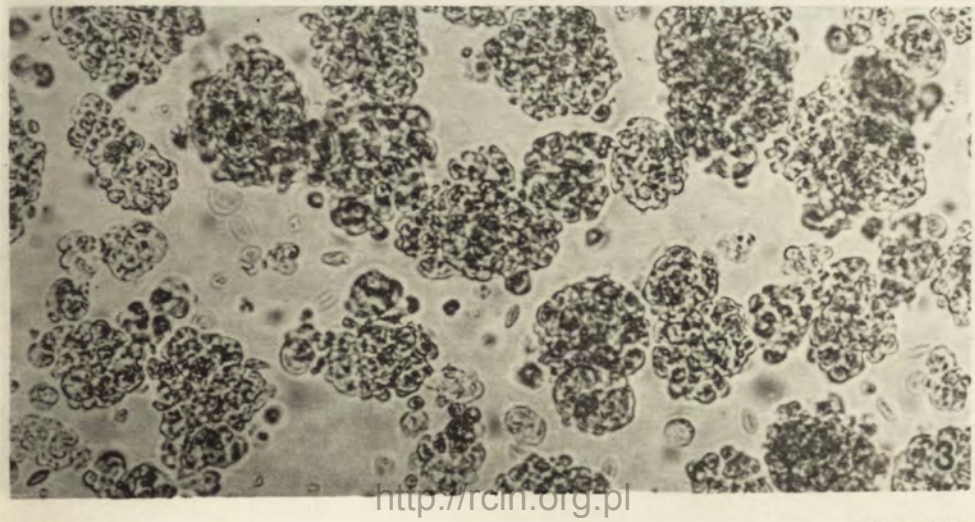
EXPLANATION OF PLATES I–III

1: Embryonic chick muscle cells remain isolated after being rotated for 4 h in the presence e of ethylenediaminetetra-acetic acid (EDTA) at a concentration of 1×10^{-4} M. Under conditioions of turbidimetric method (see Jones 1965)
2: The same cells have produced only small loosely formed aggregates after being rotated f for 4 h in the presence of mersalyl at a concentration of 5×10^{-4} M
3: The same cells have produced large well-formed aggregates in Hanks's balanced salts solutioion. Control preparation
4: Green (shown white) fluorescent staining by anti-chicken gizzard smooth muscle actomyososin γ-globulins of cells (monolayer) from the muscle tissue of 9 day old chick embryos
5: Same as 1 enlarged to show individual cells
6: Failure of fluoresced anti-chicken striated muscle actomyosin γ-globulins to stain the sarame cells
7: Failure of fluoresced γ-globulins from non-immunized rabbit serum (NIS) to stain cecells
8: Production of large well-formed aggregates by embryonic chick muscle cells after being rotatated for 24 h in the presence of anti-chicken striated muscle actomyosin (anti-PAM). Gyratory shakaker aggregation method (see Jones and Kemp 1970, Kemp et al. 1971)
9: Well-formed aggregates by same cells after being rotated for 24 h in the presence of calf serurum. Control preparation
10: Well-formed aggregates by same cells after being rotated for 24 h in the presence of non-ir-immunized rabbit serum (NIS). Control preparation
11: Production of small aggregates by same cells after being rotated for 24 h in the presence e of anti-chicken gizzard smooth muscle actomyosin (anti-GAM). Shows marked inhibitory effeffect of the anti-GAM on aggregative competence of the cells







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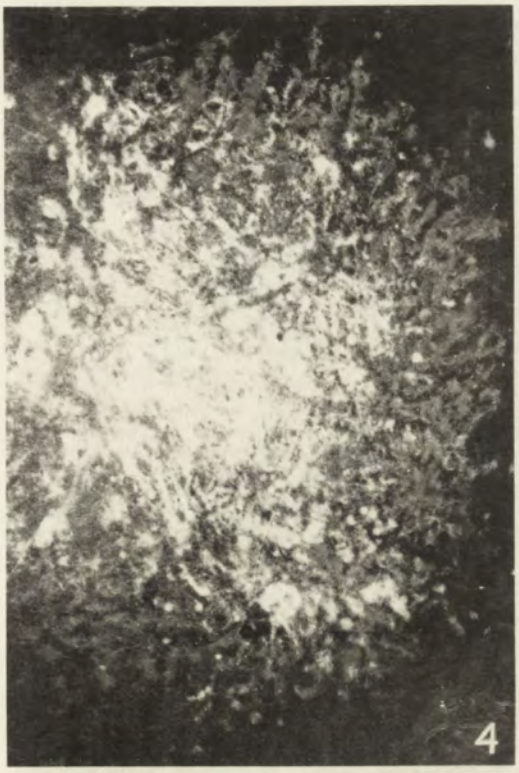

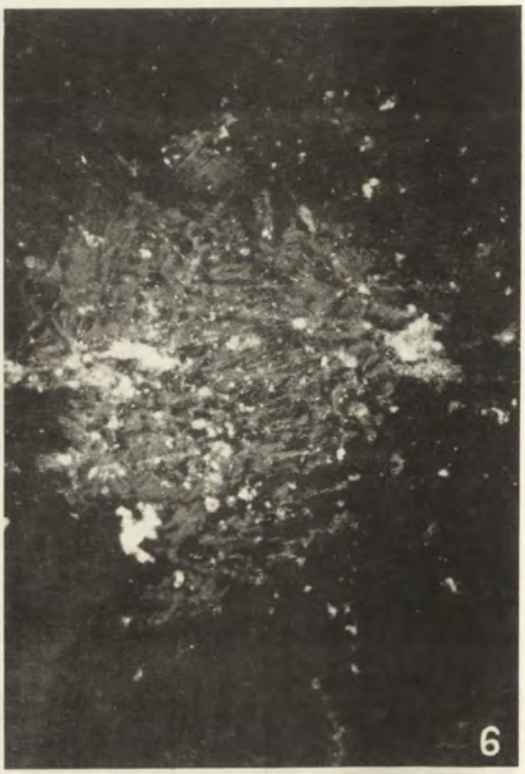

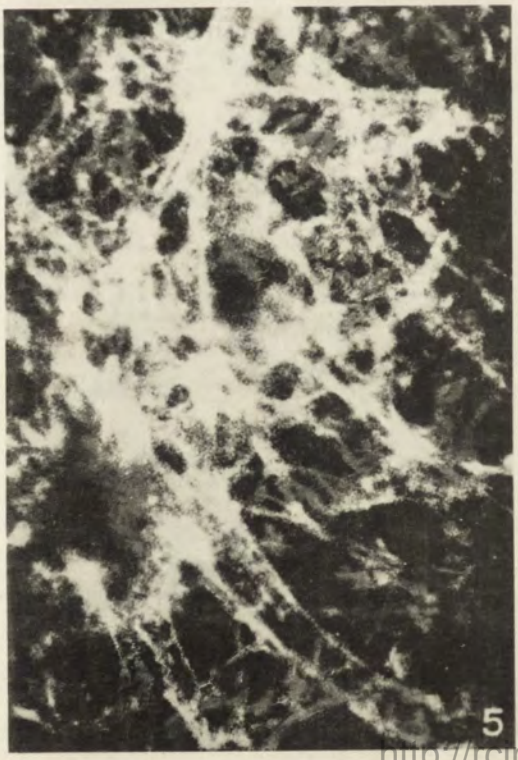

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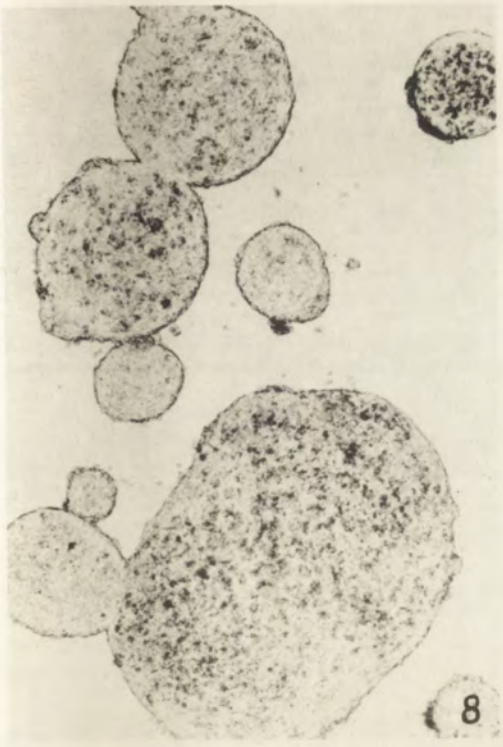

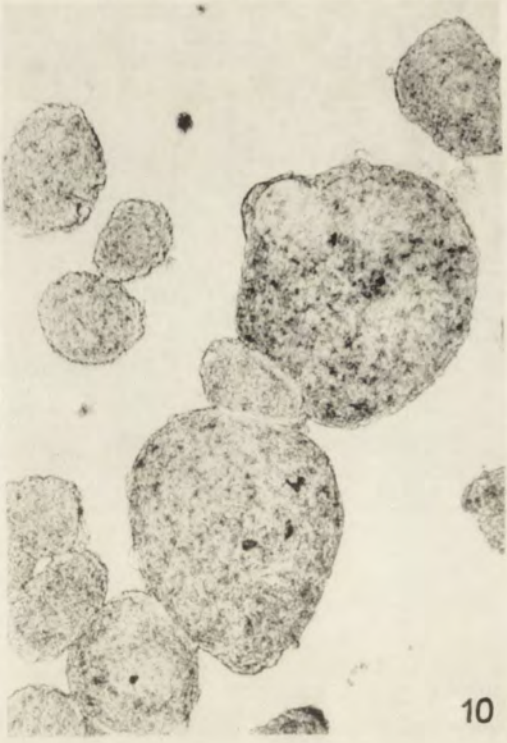

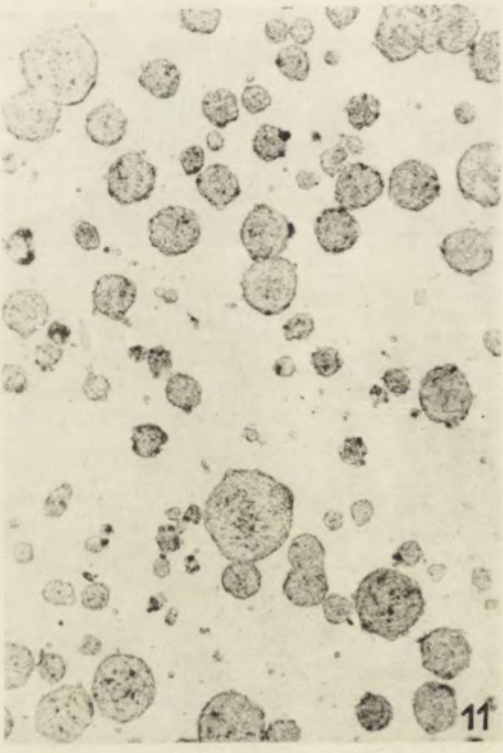

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Some aspects of the interrelation among cell locomotion, contact phenomena, and cellular multiplication

In this communication I intend to discuss some aspects of the significance of contact phenomena for a control of cellular multiplication in tissue culture conditions. During the last decade an increasing attention has been paid to the contact inhibition of cellular movements resulting from the formation of stable adhesion between surfaces of colliding cells. This interest in contact phenomena was mainly due to the observations which demonstrated that:

(1) Cancer cells or virus-transformed cells do not show or show much less extended inhibition of locomotion when being in mutual contact and they can freely crawl over the surface of other cells (Abercrombie and Heaysman 1957; Ambrose and Easty 1960, Abercrombie and Ambrose 1962, Abercrombie 1962).

(2) Parallelly with the "contact" inhibition of cellular locomotion the inhibition of cellular multiplication occurs (at least in epitheliocytes and fibroblasts). As a result the cell monolayering takes place and the population density stabilizes at relatively low values. However, cancer cells and virus-transformed cells which are not contact-inhibited in their movements do not show the "contact" inhibition of growth and multiplication either. They continue locomotion, growth, and divisions in much more crowded cultures than normal cells, which often leads to multilayering (Sachs and Medina 1961, Dulbecco 1961, Stocker and Mac Person 1961).

These two basic observations raise numerous questions concerning the dependence of the formation of stable adhesion between cells upon a character of the cell surfaces, mechanism of cell recognition, cellular locomotion, and events involved in the inhibition of cellular multiplication. All these aspects of the contact phenomena have been extensively discussed in the contemporary literature and numerous hypotheses for their explanation have put forward. In the present communication I would like to concentrate on the problem: why does the formation of stable adhesion between surfaces of cells growing in vitro, while leading to the inhibition of cellular locomotion, cause also parallel inhibition in cellular multiplication? (Question "I").

To find the answer to this question let us collect together and follow the basic facts concerning the interrelation of cell multiplication and contact phenomena.

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(1) In cell culture conditions normal cells can move over a solid surface. When two cells collide adhesion often forms at the point of contact between the two membranes. At the region of contact the local inhibition in motile activities of the cell surface layers usually occurs. Since the immobilization of the cells in contact with one another is of a very local character and is confined to the regions of stable adhesion, the cells can form new lamellipodia and start migration in new directions. As a result, at the original contact a retraction can develop which may be sufficient to break the adhesion between the cells (cf. Ambrose and Easty 1960, Abercrombie 1961, 1965, Wolpert and Gingell 1969, Trinkaus et al. 1971, Korohoda 1971). The frequency of stable contact formation depends upon a cell type. When some of the cancer cells or virus-transformed cells collide with themselves or with other normal cells the stable adhesion between the cells occurs only very rarely and the contact inhibition of their locomotion usually does not occur (cf. Abercrombie 1962, Ambrose and Easty 1960, Dulbecco 1970, Gail and Boone 1971).

(2) An increase in the density of cell population causes more frequent cell collisions and more frequent formation of stable adhesion among cells. This leads to gradual decrease in the average rate of cellular locomotion which depends upon the local cell concentration (Abercrombie and Heaysman 1953, Gail and Boone 1971).

(3) Since normal cells cannot usually use other cells as a substrate for their locomotion (Abercrombie 1965), when the culture approaches the stage of confluence the cells form a monolayer of contact-inhibited cells lining up parallel to one another. In such confluent monolayer most of the cells are fully inhibited in their locomotion (Abercrombie and Heaysman 1953, 1954, Abercrombie and Ambrose 1962, Ambrose 1968). The saturation density of cells depends upon the external conditions and primarily upon the character of the cells (Abercrombie 1962, Oldfield 1963, Clarke et al. 1970, Ceccarini and Eagle 1971). Cancer cells and virus-transformed cells which can locomote relatively freely in dense cultures show much higher saturation density than normal cells (Dulbecco 1970, Gail and Boone 1971).

(4) Contact phenomena among cells depend on the character of the cell surface. Though we do not intend to discuss here the mechanisms involved in the formation of stable adhesion among cells it should be noticed that the physical and chemical properties of the cell surface are tissue specific. In the case of cancer cells and virus-transformed cells the surface of the cell membrane definitely differs from the surface of the native cells (cf. Abercrombie and Ambrose 1962, Ambrose 1967, 1968). Normal cells when actually dividing do not show contact inhibition of movements and at this stage their surface properties resemble transformed cells (Abercrombie 1962, Fox et al. 1971). The covering of agglutinin sites on virus-transformed cells with trypsinized concanavalin A causes the restoration of contact inhibition of locomotion characteristic for untransformed native cells (Burger and Noonan 1970). On the other hand the capacity of cells to aggregate can be abolished by specific antibodies bound to the defined loci at the cell surface, as was shown in cellular slime moulds by Beug et al. 1970.

(5) The formation of stable adhesion between cell surfaces often results in the so-called "coupling" phenomenon, discovered by Kanno and Loewenstein 1964, originally in the salivary gland cells of *Drosophila flavorepleta* larvae. This phenomenon involves a great decrease in the electric resistance of contacting membranes and a simultaneous great increase in their permeability. In the functionally coupled membranes the increase in permeability was observed not only for ions but also for fluorescein and even proteins of low molecular weight. The cancer cells, as has been found in several cases, in contrast to normal cells often do not show the functional coupling when in contact (cf. Loewenstein et. al. 1967, Kanno and Loewenstein 1966, Martinez-Palomo 1970, Burton 1971).

(6) The normal cells when cultured in vitro divide at the rate inversely correlated with the density of cell population, at least above rather low densities. In one and the same culture the rate of cellular multiplication depends on the local density of cell population per surface area of the substratum (Pace and Layon 1960, Schultz and Mara 1968, Rein and Rubin 1968, Zetterberg and Auer 1970).

(7) Full inhibition of cellular locomotion in confluent cultures causes the inhibition of cell multiplication. The transformed cells which are not inhibited in locomotion by mutual contacts are not inhibited in their multiplication. Covering of their surface by Concanavalin A causes not only restoration of contact inhibition of locomotion but also the inhibition of cell divisions (Burger and Noonan 1970). The cells immobilized in a monolayer do not divide because they are inhibited at the end of the G1 phase of their life cycle and cannot enter the S phase, i.e. they cannot start DNA replication (Nilausen and Green 1965, Clarke et al. 1970, Dulbecco 1970, Sefton and Rubin 1970, Zetterberg and Auer 1970).

(8) For stimulation of the cells originally contact-inhibited to DNA synthesis and divisions it is sufficient to provide the additional surface of solid available for cell locomotion. It can be easily done by wounding the confluent monolayer of cells. Only the cells which start to migrate into the wound start the synthesis of DNA and multiplication. Agitation or flow of the medium does not stimulate the cells immobilized in the monolayer (Todaro et al. 1965, Vasiliev et al. 1969, Dulbecco 1970, Clarke et al. 1970).

The facts so far cited lead to the conclusion that the contact inhibition of cell multiplication is correlated with the contact inhibition of cell locomotion. This parallelism was always interpreted in terms of the "informational coupling" of the adhering cells (cf. item 5). It was postulated that macromolecules or ions passing from one cell to another carry the information which is responsible for switching back or forth the cellular multiplication (cf. Ambrose 1967, Loewenstein 1968, Wolpert and Gingell 1969, Burton 1971). All these considerations postulated the necessity of the hypothetical substances, the exchange of which from cell to cell controls the cell multiplication. However, there are numerous facts which strongly suggest that an exchange of the specific substances between contact-inhibited cells is unnecessary for the "contact" control of cellular multiplication and DNA synthesis

in contact-sensitive cells. The significance of the functional coupling between cell membranes can be much greater for the control of the cell differentiation than of the cell multiplication. The inhibition of DNA synthesis and thus divisions in the cells which show the contact inhibition of locomotion seems to be rather a secondary effect of the cell immobilization. This supposition can be supported by conclusions derived from the analysis of observations quoted above and from numerous experimental results.

(9) As it was pointed out by Wolpert and Gingell 1969, it is difficult to imagine why an exchange of substances between similar cells should result in a considerable change of their metabolism.

(10) If the specific chemical substances were responsible for the inhibition of DNA synthesis in the contact-inhibited cells, these effects should be more permanent than it is observed (cf. items 2, 6, and 8). The originally "coupled" cells should show identical behaviour independently of whether one of them locomotes or not but the observations of the cells lying at the border of the wound made in the confluent monolayer contradict it (cf. 8).

(1) The inhibition of cellular locomotion by stable contact formation is primarily phenomenon and does not seem to be caused by the specific molecules or ions passing from one contacting cell to another (cf. arguments of Trinkaus et al. 1971). It results rather from the local inhibition of motile activity of the superficial cell layers (cf. item 1). If the specific chemical substance were responsible for the inhibition of the DNA synthesis in the contact-inhibited cells, it should be the substance which acts independently of cell motile activities. This is, however, in obvious discrepancy with the facts noticed in points 2, 6, 7, and 8.

(12) The contact inhibition of cell locomotion and multiplication can be observed only in the anchorage-dependent cells. It has been repeatedly observed since the beginning of the work with cell cultures in vitro that almost all types of normal cells need to attach to solid substrata and spread on them for normal growth and divisions (Willmer 1965, Barski and Belehradek 1968). The anchorage dependent cells do not grow or multiply when in suspension. To induce their growth and multiplication it is sufficient to allow them to spread over solid substrata and start locomotion. No chemical treatment is necessary to stimulate these dramatic changes in cell metabolic activity (cf. p. 8). Recently Clarke et al. 1970 have found that the anchorage-dependent cells can synthesize DNA even in suspension but at the rate many times lower than when actively locomoting on the solid substratum.

(13) Since the anchorage-dependent cells suspended in fluid media grow and divide at practically negligible rate we made the following simple experiments (Korohoda in prep.). Chick embryo fibroblast-like cells after trypsinisation of primary cultures were incubated in suspension for 24 h before sowing on the glass. The cells were incubated in siliconized glass and gently shaken in medium No. 199 with the calf serum, at 37°C. Then the cells were transferred with the original, conditioned medium to 20 culture vessels in which they sedimented, spread on glass and started locomo-

tion. Every one hour one vessel was fixed, stained with carbol fuchsine, and the mitotic index was estimated. The experiment was repeated four times, and it was always observed that the cells pretreated in this manner divided later in a semi-synchronous manner. The mitotic index which for the first 8 h was fairly low reached the maxima at 11 and 17 h after cell spreading (Fig. 1). The first maximum was more pronounced and the index reached the value of aprox. 20%. The time lapse observations of these cells showed that the cells which divided at 17 h after the spreading

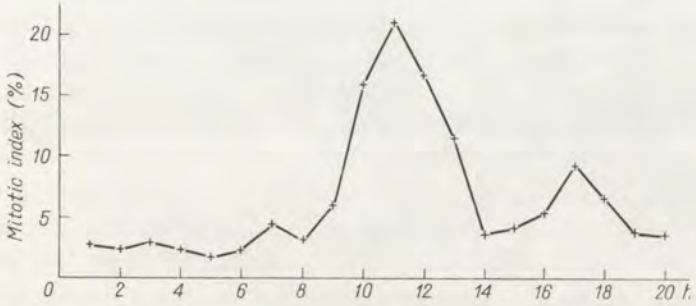

Fig. 1. Mitotic index as a function of time in chick embryo cells which had been incubated in suspension for 24 h before they were transferred to culture vessels. Cummulative data from four experiments

were just during mitosis when transferred from the suspension. Some of the cells inhibited at the beginning of the mitosis were later unable to complete division for subsequent 20 h, though they were apparently alive and showed fairly vigorous "amoeboid" activity. An analysis of changes observed in the mitotic index with time in the cultures of the cells which were earlier preincubated in suspension suggests that the immobile cells in suspension became arrested mostly at the same phase of their life cycle (at the end of the Gl phase or at the beginning of the S phase), as the cells immobilized by the contact inhibition of their locomotion. In both cases the inhibition is reversible – the contact with a free surface of solid substratum and the stimulation of cell locomotion are sufficient to enable the cells to multiply.

(14) In the processes of the wound healing in vivo (Szent-Györgyi 1965), in developing embryos and in the organ cultures in vitro it was often observed that the rate of cell multiplication is correlated with the cellular locomotion. For example, Gierthy and Rothstein 1971 studied the organ cultures of leaopard frog lens and came to the conclusion that: "Whatever its cause the migration phenomenon provides us with a sure morphological marker for the prospective site of heavy mitotic activity".

The above-quoted observations (cf. 2, 6, 8, 11–14) strongly support the suggestion that in the anchorage-dependent cells the inhibition of the synthesis of DNA and hence of cell multiplication in contact-inhibited cells results primarily from the in-

hibition of the cell active locomotion. Thus question "I" can be put in the form: why do the phenomena which influence cellular locomotion simultaneously influence the rate of multiplication of cells by control of their DNA synthesis (Question "II"). To find the answer to this question let us consider some facts with regard to the processes of DNA replication and completion of the S phase by cells, and to the processes which control cellular metabolism.

(15) The progress of eucariotic cells through the full sequence of the phases of cell life cycle is determined by intrinsic cellular factors. However, the biochemical processes characteristic for particular phases seem to differ in their requirements concerning substrates and a general level of cellular metabolism. The external factors which are known to influence the normal progress of cells through their life cycle and hence the cell multiplication can be expected to exert their effect by making selectively impossible the realization of such intrinsic biochemical events without changing the cell viability.

There are numerous recent data which point to a conclusion that the DNA replication is associated with a relatively high rate of the energetic metabolism, i.e. glycolysis or respiration depending on a particular cell type. It is well known that the inhibitors of the carbohydrate metabolism inhibit cell multiplication reversibly (cf. Ely et al. 1953, Ball et al. 1957, Bekesi and Winzler 1970). Malamud and Baserga 1968 found that the stimulation of DNA synthesis in the isoproterenol-stimulated salivary glands of rats and mice is dependent on and preceded by the activation of the carbohydrate metabolism. A similar conclusion concerning the initiation of DNA synthesis in the cells of pea root meristems was reached by Webster and Van't Hof 1969, as they found that the entry of Gl cells into S phase was reversibly prevented by carbohydrate starvation, anaerobiosis, and treatment with *2,4*-dinitrophenol. Polgar et al. 1968 studied the transformation of the lymphocytes by phytohaemagglutinin (PHA) and concluded that in these cells the stimulation of DNA synthesis is dependent on and preceded by an increase in the glycolytic activity. Robbins and Morrill 1969 found that in the synchronous suspension cultures of HeLa S3 cells the rate of respiration increases significantly in the S phase of the cell life cycle. These observations seem to make plausible the assumption that also in the cells showing the phenomena of contact inhibition and anchorage dependence the replication of DNA can be dependent on the rate of their energetic metabolism. This interrelation can be expected to be fairly common, at least in the cells in which the DNA replication can be relatively easily switched back and forth by external factors.

(16) The rate of the cellular energetic metabolism, i.e. glycolysis and respiration, is controlled not only by the presence of substrates, but also, and this seems to be more important, by the ADP/ATP ratio in the cytoplasm (cf. Chance 1961, 1965, Whittam 1964, Atkinson 1965). This ratio can be influenced, among others, by the processes in which ATP is split.

(17) The cellular locomotion is an active process and occurs due to the contractile

reactions of the cytoplasm. The presence of the contractile proteins similar to the acto-myosin complex of vertebrate muscles has been disclosed in the cytoplasm of not only amoebae and slime mould plasmodia but also in the fibroblasts. These proteins have the capacity to split the ATP molecules and to convert the chemical energy into kinetic energy (Hoffmann-Berling 1956, Buckley and Porter 1967, Keyserlingk 1968, Schäfer-Danneel and Weissenfels 1969). Thus the contractile reactions of the cytoplasm can be expected to influence the ADP/ATP ratio.

(18) Weiss 1958, Abercrombie 1965, Ambrose 1962, 1967, 1968, Carter 1967, Korohoda 1971, Trinkaus et al. 1971, and many others showed that the cellular movements and cell social behaviour are determined by contact relations among the cells themselves and between the cells and substratum. In amoebae and in slime mould plasmodia, i.e. in the objects in which movements are most highly organized, the polarization of cellular locomotion and protoplasmic streamings are determined by the polarization of cell membrane activities (cf. Korohoda et al. 1969, 1970, Korohoda 1970, 1972). Like in muscles also in other motile cells the contractile reactions of the cytoplasm appear to be controlled by the activity of the cell membrane. Therefore one can postulate fairly safely that the contact phenomena which switch back and forth cellular locomotion can exert an influence upon the ATPase activity of cytoplasmic contractile proteins and hence upon the ADP (ATP ratio) in the cytoplasm.

(19) From the facts observed at points 16, 17, and 18, one could predict that the rate of the energetic metabolism should be much higher in the actively locomoting cells than in the immobile cells. In muscles, it was observed and studied in detail that the excitation and contractile processes are correlated with the increased rate of the energetic reactions involving the turnover of adenine nucleotides (cf. for example Chance 1965). However, up to 1971 the measurements of the rate of cellular respiration were usually carried out with cell suspensions or tissue slices and there was no information concerning the interrelation of cellular locomotion and cell energetic metabolism (cf. Paul 1965). Using the microrespirometer developed by Zurzycki and Starzecki (1971), it appeared possible to measure the respiration of cells suspended in a fluid medium, spread on the glass surface but contact inhibited, and spread on glass and actively locomoting. Simultaneously, it was possible to observe the cells microscopically. The measurements were always carried out on cells from one suspension in these three types of conditions. An increase in the available surface area of solid substratum necessary for cell locomotion was obtained by putting small twists of glass wool into the sample (for details cf. Korohoda 1971 a, b). The results of measurements carried out with chick embryo fibroblast-like cells showed that the respiration in actively locomoting cells was approx. 5 times higher than in the cells remaining in suspension and aprox. 6–10 times higher than in the contact-inhibited cells (Table 1). These significant differences resulted from various contact relations in particular samples and not from chemical differences

Table 1

The respiratory rate in chicken embryo fibroplast-like cells in μl O_2 (million cells) h in relation to motile activity of cells (from Korohoda 1971)

Immobile fibroblasts:		Fibroblasts actively locomoting on surface of glass
remaining in suspension	attached to glass but contact-inhibited	
4.16	2.68	13.88
4.47	2.35	16.01
3.60	2.47	14.32
5.40	2.53	21.90
4.35	1.24	13.90
2.92	1.57	18.04
3.62	2.38	13.20
4.40	1.55	29.25
3.77	1.85	12.85
4.00	3.89	35.40
4.07±0.66 (SD)	2.35±0.85 (SD)	18.87

in the medium. In other words, the experimental results confirmed the expected relation between the cell locomotion and cellular energetic metabolism.

One point, however, seems to need explanation. A difference in the rate of energetic metabolism between locomoting and immobile cells was found to be much higher than it could be predicted from the calculations of the energy of cell movements (Wolpert 1965). But all these calculations take into account only the energy necessary for protoplasmic streamings and for changes in the cell shape. In locomoting cells much more energy is needed to break the contact between the cell surface and substratum than for changes in the cell shape and endoplasmic streamings. In large amoebae the forces necessary for induction of a reversal in the direction of the endoplasmic flow are apıox. 3 times lower than the forces necessary for detachment of the cell from glass (Kanno 1964). In the locomoting fibroblasts which are much more strongly attached to substratum the energy is probably used mostly for the break of adhesion of the cell ventral surface to glass. What is more, a primary increase in the rate of energetic metabolism due to the induction of cell locomotion can stimulate other metabolic reactions, including synthesis of macromolecules. Some of such reactions can be expected to occur only when a certain minimal level of energetic metabolism and of the rate of adenine nucleotide turnover is overstepped. Since these reactions have endoergic character and are accompanied by ATP hydrolysis they can modify further the ADP (ATP ratio) and hence the rate of the energetic metabolism.

(20) From the facts cited at points 15 to 19 one can conclude that the chain of events leading from the phenomena occurring at the cell surface to the processes of DNA replication should include: cell membrane activities modified by contact phenomena (a), the exchange of ions between the cytoplasm and environment

dependent on them (b), contractile reactions of the cytoplasm and cellular locomotion associated with ATP hydrolysis (c), changes in the ADP/ATP ratio which controls the rate of the cell energetic metabolism and ATP production and thus the rate of the adenine nucleotide turnover (d), the processes of the macromolecular synthesis including the DNA replication (e). The postulate of the above interrelations offers an outline of the answer to question "II" and suggests future experiments which should aim at more detailed knowledge of particular linkages of the postulated chains if events.

(21) The conclusion derived from the above-presented consideration of the known facts concerning the frequently observed correlation between the cellular locomotion and multiplication do not exclude a possible influence of various chemical substances on cellular multiplication and the DNA synthesis. On the contrary, they encourage the expectation that chemical substances can influence the DNA replication (in the living organisms the undividing cells are usually inhibited at the end of C1 phase of the cell life cycle – cf. Monesi 1969) at least in four general ways.

The presence or the absence of chemical substrates for "normal" cell metabolism can in some cases constitute the factor deciding about the rate of cellular multiplication. For example, the lack of CO_2 can inhibit both the energetic metabolism and the synthesis of nucleotides nacessary for DNA replication (Chang et al. 1961).

The chemical factors which influence the cellular locomotion or modify the stabilization of contacts among cells will indirectly influence the energetic metabolism and cell multiplication. Such chemical substances can act in a very specific way on particular cell types and need not penetrate the cells to exert their effect on cell metabolism (cf. results of Yeh and Fischer 1969). Likewise the substances modifying the processes of active transport at the cell membrane change the rate of energetic metabolism (cf. Whittam 1964) and can be expected to influence the rate of the DNA replication. These substances again need not penetrate the cell membrane (cf. results of Quastel and Kaplan 1970, Lindahl-Kiessling and Mattsson 1971, and Polgar et al. 1968).

The substances which directly influence the energetic metabolism are known to inhibit reversibly the rate of DNA synthesis and cell multiplication (cf. p. 15).

And there are specific substances which interact directly with the metabolic pathways of the DNA replication. These substances are involved probably in the inhibition of the DNA synthesis in specialized cells characterized by a high rate of general cellular metabolism, such as in nerves and muscles. Also the natural sequence of the particular phases occurring during the cell life cycle must be specifically controlled by intracellular factors.

The considerations presented in this communication concern only a limited range of the processes which control the cellular multiplication and are relevant only to the cells in which the inhibition of the DNA synthesis can be relatively easily reversed. This inhibition in such cells seems to be abolished by factors which stimu-

late more or less specifically the cellular energetic metabolism and the postulation of the presence in the contact-sensitive cells of specific inhibitors of DNA synthesis exchangeable from one cell to another does not seem to be sufficiently justified.

Summary

Interrelations between cell locomotion, contact phenomena, and cellular multiplication have been discussed in the light of available literature and the author's own recent experimental results. It was concluded that the inhibition of DNA synthesis in contact-inhibited cells results primarily from the inhibition of cellular locomotion and does not require the presence of specific inhibitors of DNA synthesis which could be exchangeable from one cell to another. The working hypothesis which aims at explaining why inhibition of cellular locomotion results in a parallel inhibition of DNA synthesis in contact-inhibited cells is formulated and discussed.

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Motile activities in chick embryo fibroblast-like cells (16 mm movie, 15 min of projection)

The black-white movie showing movements of separate chick embryo fibroblast-like cells and cellular aggregates attached to glass fibres suspended in a fluid medium was demonstrated. The results of the analysis of this movie were published elsewhere (cf. Korohoda W. 1971: Folia Biologica, 19, 191–199) and therefore they are not included in the proceedings of this Symposium.

Presented at Symposium "Motile Systems of Cells", Kraków, August 3-7, 1971.

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Polytomella agilis aggregations at ultra-high population densities [2]

Polytomella agilis, other flagellates, and ciliates have been observed to form aggregates which generate complex macroscopic patterns (see reviews by Gittleson and Jahn 1968 a and Winet 1970). Such activity by microorganisms has been termed "pattern swimming" to indicate that these motile cells form aggregates which are dependent upon swimming behavior rather than cell body attachments. Theoretical calculations and experimental evidence points to the importance of hydrodynamic associations (Gittleson and Jahn 1968 b).

Until now studies of pattern swimming have been made on cultures containing about 100 000 to 1 million cells/ml. This report describes pattern swimming by *P. agilis* in ultra-high population densities as high as 278 million cells/ml. Overcrowding of cells inhibits the formation of patterns which are characteristically observed at lower densities but induces patterns of a different geometric configuration.

Methods

Polytomella agilis (Doflein, 1916) were cultured for experimentation in 1 L of medium in 2 L Erlenmeyer flasks. Each flask was inoculated with 9 ml of a 2–3 day old stock subcultured in 125 × 16 mm screw top test tubes. Aseptic techniques were employed to maintain axenic cultures. All cultures were grown at room temperature of 19–23°C.

The medium was composed of 1 g sodium acetate, 2 g yeast extract, and 1 g tryptone per 100 ml of glass distilled water and was autoclaved at 16 p.s.i., 121°C for 16–20 min. All glassware was washed with alconox cleanser, rinsed eight times with tap water, twice with dilute nitric acid and twice with glass distilled water.

Ultra-high population density samples were obtained with some modification according to the basic technique previously described by Gittleson, Woodruff and Hoover 1970 for mass accumulation of protozoa. 500 ml of a 48 h culture were placed into a 500 ml Sedgewick-Rafter graduated funnel. The bottom end of the funnel was plugged with a number 0 rubber

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stopper that had been shortened to allow easy penetration of an 18–20 gauge hypodermic needle. *P. agilis* were accumulated in the bottom of the Sedgewick-Rafter funnel for 30–45 min and removed through the hypodermic needle into a 1/2 ml glass syringe.

Cell counts of stock cultures and ultra-high population density samples were made using a Coulter electronic particle counter, Model B (Coulter Inc. Hialeah, Florida). Isoton manufactured by Coulter Inc. was used as a diluent. *P. agilis* remained intact and viable in Isoton. Preliminary cell counts made with the Sedgewick-Rafter method (Scherbaum, 1957) were within 5% of the Coulter counter values.

The ultra-high population density samples were observed in a 2.57 cm diameter flat-bottomed glass chamber (Zeiss plankton counting chamber) at various depths. This chamber was covered with a glass disc to prevent evaporation and was removed during photography. No difference was noted in the aggregate pattern covered or uncovered. Pattern swimming was disrupted with streams of air blown across the chamber.

Pattern formation was photographed with a Nikon 35 mm camera equipped with Nikkor number 1 and 2 close-up lens. The camera was positioned directly above the chamber which rested on a glass platform. A 15 watt lamp placed beneath this platform was used for illumination during photography. Pattern formation occurred in the same way with dim overhead illumination or in the dark as tested by random intermittent lighting. Kodak Tri-X negative film was used. Temperature of the air conditioned room varied less than 0.1°C during any one experiment. Between 19° and 23°C no changes in the pattern were observed.

Results

Ultra-high population densities of *P. agilis*, as viewed on the horizontal plane, formed dense masses of aggregated cells in the form of lines and dots interdispersed by the less densely populated medium (Plates I 1–6 and II 7–12). Within 5 sec after complete disruption of an existing pattern there was noticeable reformation. A more complete pattern formed in 15–20 sec. Pattern formation time did not vary with change in chamber size, shape or light intensity.

Patterns formed in depths of medium as low as 0.3 mm and as great as 4.0 mm. The range of cell numbers in the ultra-high population density was 105–278 million/ml. The particular experiment illustrated in Plates I and II contained 170 million cells/ml. The depth in this case was 0.3 mm.

The initial pattern consisted of short chains and circular nodes of aggregated cells (Plate I 1–6). After about 100 sec the linear pattern within the central area of the chamber was replaced by a nodal system. These centrally located nodes of aggregated cells were distinct from radially arranged short chains at the periphery (Plate II 7). In Plate II 8 (180 sec) the circular groups began to merge into chain-like configurations. This process proceeded (Plate II 9–12) until finally at 300 sec nearly all of the pattern was linear. Patterns remained in a chain-like convolution beyond 5 min. The sequence of pattern swimming events involving a mixture of nodes plus chains, then primarily nodes and finally predomination of chain-like formations occurred again after complete disruption.

Discussion

The contrast between pattern swimming by *P. agilis* at low and ultra-high cell numbers/ml is summarized in Table 1. Clearly, cell number/ml is a major factor associated with (a) initiation of patterns (the pattern swimming behavior of all species reported to date typically occurs only above a minimum cell number/ml); (b) pattern configuration; (c) the time necessary for pattern reformation after disruption and (d) a minimum depth of medium required for pattern formation. This

Table 1

Comparison of pattern swimming by *Polytomella agilis* at low and ultra-high population densities

	Low population densities	Ultra-high population densities
Population densities at which patterns form (cells/ml)	200 000 to 1.5 million	105 to 278 million
Pattern configuration	pattern is initially circular in circular chamber becoming asterisk in shape and finally a complex network-pattern is always linear	pattern initially develops over entire surface as nodes and convoluted chains
Pattern formation time	varies with cell number/ml from 20 to 90 sec	about 5 sec at all cell numbers
Depth of medium	2 mm lower limit	0.3 mm lower limit

influence of population density further supports the concept of a hydrodynamic association of flagella among closely swimming cells as an underlying mechanism of pattern formation (see Gittleson and Jahn 1968 a for detailed discussion of other evidence). Viz. as *P. agilis* come closer together flagellar motion of one cell affects another in the same way that polystyrene spheres in the medium were shown to be pulled in toward the cell body (Gittleson and Jahn 1968 b). In the case of spermatozoa it has been shown by direct measurement that their degree of closeness effects cell orientation and that a reduction in sperm density decreased the sperm order (Reynolds and Rothschild 1962).

The ultra-high population densities of *P. agilis* creates a statistically greater chance for flagella interaction and thus for an ordering among cells. This would account for faster formation and for an alteration in pattern configuation at ultra-high densities as compared with lower densities. Gittleson and Jahn 1968 a observed that pattern swimming in the low cell number/ml range involves a reorientation of randomly swimming *P. agilis* at the wall of the container. A different pattern configuration probably results in the populations of higher cell number because they interact before a boundary effect takes place.

Summary

This report describes the aggregations of motile *Polytomella agilis* in ultra-high population densities (105–278 million cells/ml). Patterns varied from a mixture of nodes plus chains, then primarily nodes and finally stabilized into a predomination of chain-like formations. These events occurred over a period of about 300 sec after complete disruption of the aggregations. In comparison with pattern swimming at low population densities (200000 to 1.5 million cells/ml), cell number/ml is found to be a major factor associated with initiation of patterns, pattern configuration, pattern formation time and a minimum depth of medium.

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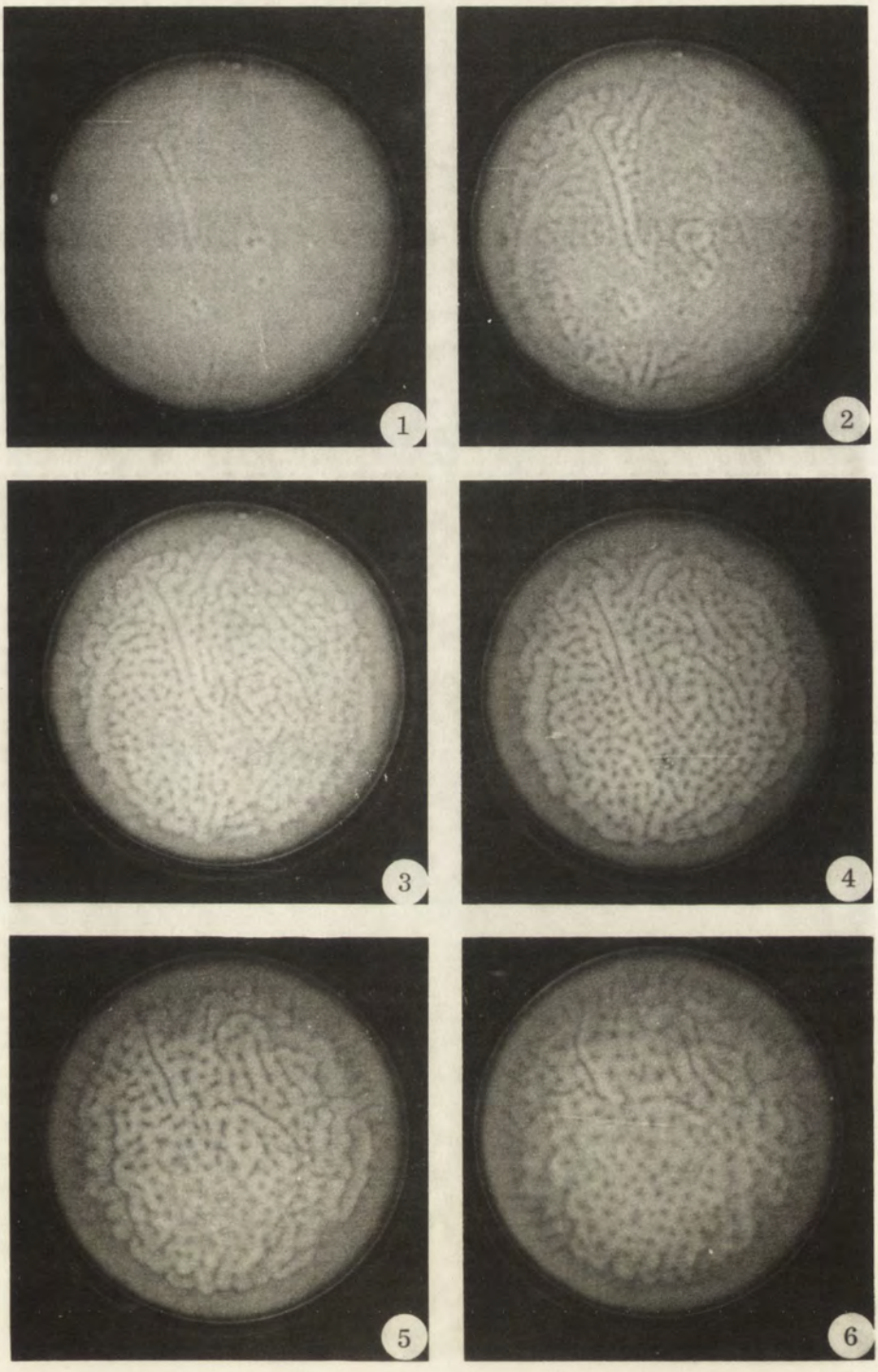
EXPLANATION OF PLATES I–II

1–12: A sequence of photographs of *Polytomella agilis* aggregate formations taken from about 5 sec after complete disruption of an existing pattern (phot. 1) to about 100 sec (phot. 6), and about 130 sec (phot. 7) to 300 sec (phot. 12). Population density is 170 million cells/ml. Magnifiction is 2×



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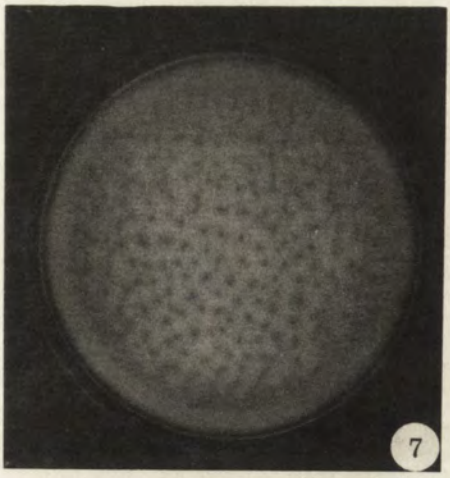

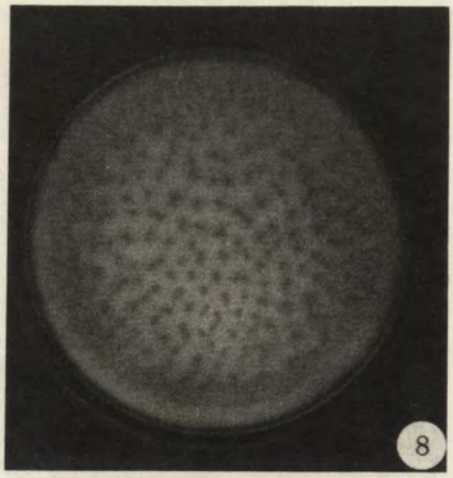

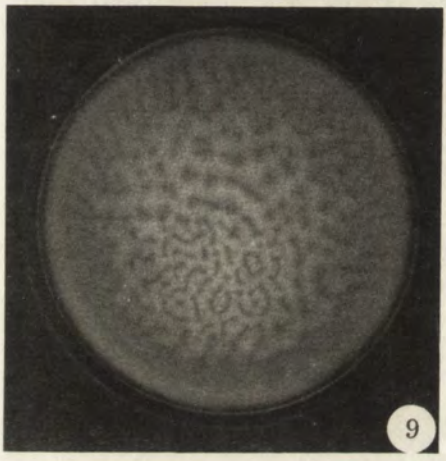

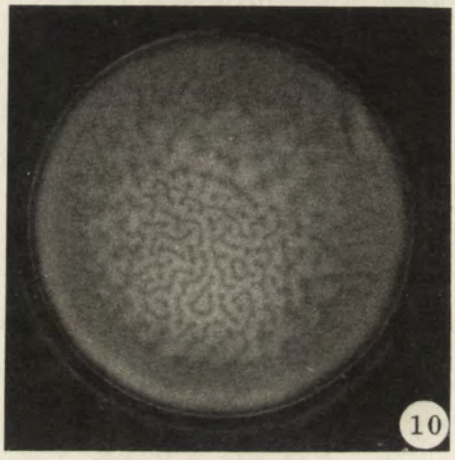

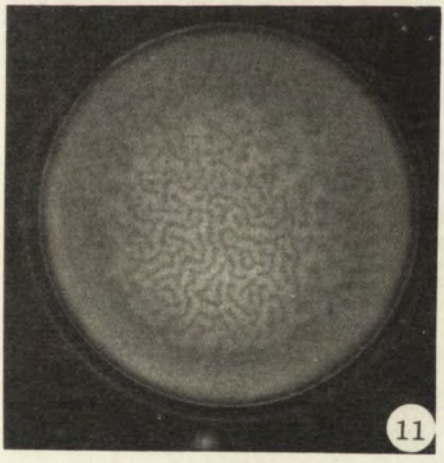

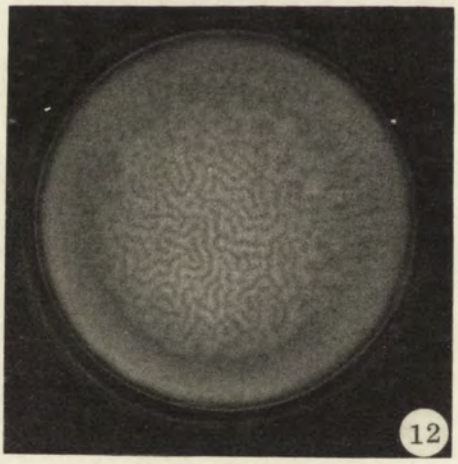

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Perception of light direction in oriented displacements of cell organelles

Movement of cell organelles inside a plant cell is a very common feature. In internode cells of *Chara* and *Nitella*, the inner cytoplasm is steadily moving along the outer cytoplasm, taking with it smaller cytoplasmic inclusions; still more impressive is the rotation streaming or cyclosis in *Vallisneria* or *Elodea*, where also chloroplasts are moving with the cytoplasm. In these latter plants, cyclosis can be induced or intensified by light which in this way is a triggering or controlling factor of that movement (cf. Seitz 1964, 1967). However, as is evident from that type of movement, there is not any orienting effect of light at all.

Besides these unoriented protoplasmic movements, there are also movements of cell contents which are oriented by light. As an example we know the accumulation of protoplasm in the coenocytic alga *Vaucheria* at those regions which are illluminated by light (Fig. 1). This response is a special case of a much more common

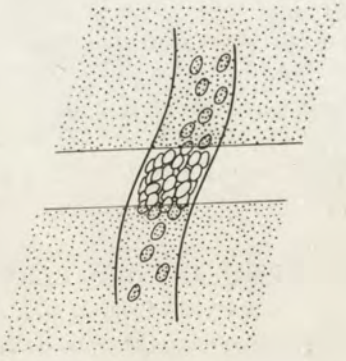

Fig. 1. Part of the coenocytic filament of *Vaucheria* with chloroplasts accumulating in that region which has been given white light. Dotted regions darkened

response, the displacement of chloroplasts in the cell in relation to light intensity and light direction (cf. Haupt 1959). Zurzycki (1962 b, 1965, 1967) has done much important work concerning these movements, especially in *Lemna* and *Funaria*, and the main features are depicted in Fig. 2, showing the alga *Vaucheria*

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

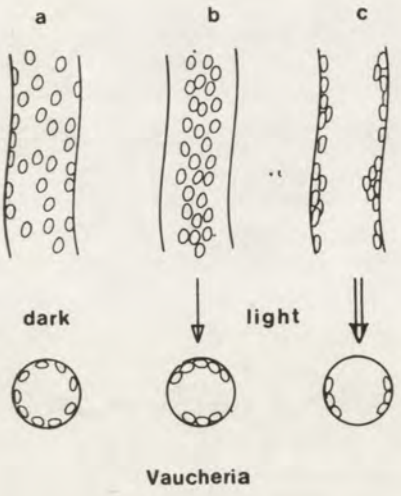

Fig. 2. *Vaucheria* surface view (top row) and cross-section (bottom row) with chloroplasts in dark (a) low intensity (b) and high intensity light arrangement (c). Light direction is shown by arrows

in darkness and in low and high intensity light respectively, given from above and in cross-section.

These orientation movements of chloroplasts which will be the topic of the present paper can be regarded as indicators of a more general displacement of cytoplasm, taking the chloroplasts with it passively. Whether this is true, or whether chloroplasts behave actively in a non-moving cytoplasm, will not be decided (cf. Zurzycki 1962 a).

In order to avoid too many complications, the following will be restricted to the so-called low intensity movement, i.e. that type of displacement which results in an arrangement of chloroplasts enabling them to photosynthesize maximally ("b" in Fig. 2); this means accumulation at those regions at which light absorption is highest. Accumulation of chloroplasts in relation to the light direction, then, requires transformation of unidirectional light into a gradient of light absorption in the cell. We have to ask, therefore, how this transformation is brought about, and this, of course, is the question of the mechanism of measuring the light direction by the cell.

Taking into account structure and physical properties of a cell, four mechanisms can be imagined (Fig. 3, cf. also Haupt 1965):

(1) Whenever the optical density of cell contents is high, much light will be absorbed on the way from front to rear, thus being attenuated strongly before reaching the rear. As a result, a strong front-to-rear gradient will be established.

(2) Whenever a more or less transparent cell has a higher refractive index than the surrounding medium, this cell will act as a collecting lens and the light will be focused to the rear. Thus, a rear-to-front gradient will be established. Chloroplasts accumulating at the rear, i.e., farthest from the light source, point to operation of that mechanism (Fig. 4, *Hormidium*).

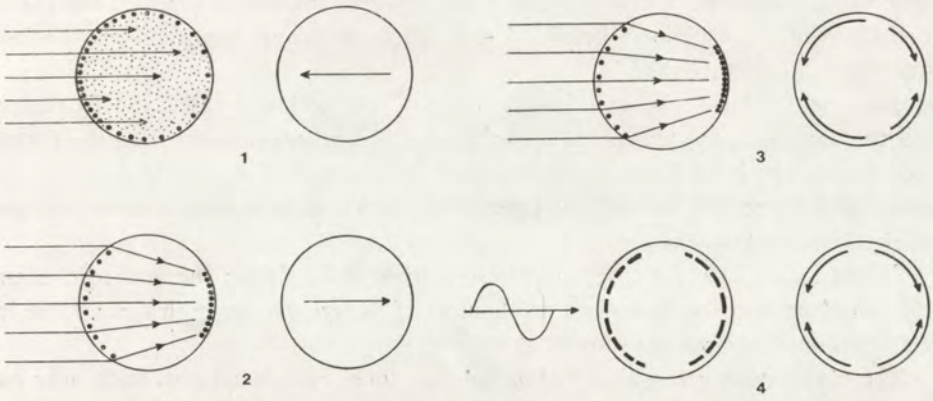

Fig. 3. Intracellular gradients of light absorption, brought about by unilateral light. On the left, photoreceptor molecules are shown near the cell surface by dots or dashes, the density of which demonstrates qualitatively regions of high and low light absorption, respectively. On the right, absorption gradients are shown by arrows. The attenuation mechanism (1) the refraction mechanism (2,3) and the dichroism mechanism (4) of measuring light direction are given. Small dots in (1) are shadowing substances by which the light is attenuated

Both the attenuation and the lens mechanism will give the cell unambiguous information about the light direction.

(3) Besides the lens effect, light refraction also results in a dark region near the flanks which will be by-passed by the light. Thus, a front-and-rear-to-flank gradient will be established.

(4) Finally, whenever photoreceptor molecules are oriented in a dichroic pattern, having their vector of main absorption parallel to the cell surface, unidirectional

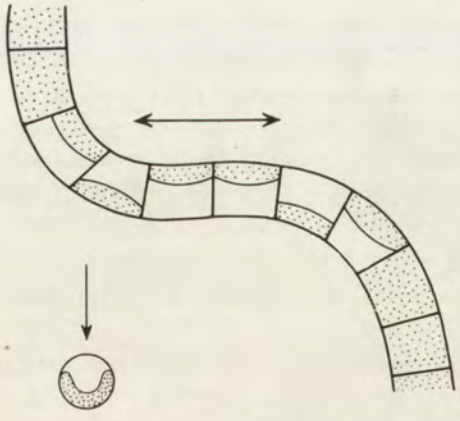

Fig. 4. *Hormidium* with chloroplasts in different positions. The filament has been irradiated normal to the plane of the paper with polarized low intensity blue light, the electrical vector vibrating according to the arrow. Notice that chloroplasts are arranged in low intensity position (inset) only in those cells which are oriented perpendicularly to the E-vector or nearly so

light will be absorbed much more at front and rear than at the flanks; the reason for that will be explained below. Thus, again, a strong front-and-rear-to-flank gradient will be established.

However, it has to be mentioned that both of the latter cases, the by-passing and the dichroism mechanism result in quite different information than the former two: though the light direction is measured, no decision is made which end of the vector is the tip; this means that light from exactly opposing directions will give nearly the same gradients.

In the following, two cases are referred to in more detail: the lens mechanism of *Hormidium* and the dichroism mechanism of *Mougeotia*. In both cases some interesting complications are shown.

The filamentous green alga *Hormidium* has short cylindrical cells with only one big chloroplast, covering about half of the cell periphery (Fig. 4). In unilateral white light, this chloroplast goes to the rear of the cylinder, thus suggesting the lens mechanism is in operation. This is true if the alga is illuminated in air (of course in moist atmosphere to avoid drying). We have tried to prove the lens mechanism in the classical way, by immersing the cells in paraffin oil. The refractive index of this oil is much higher than any known cell constituent. A transparent cylindrical cell, therefore, has to act as a diverging lens and more light can be absorbed at front than at rear. We made this experiment with *Hormidium* and, indeed, we found reversion of the response, compared with the response in air (Fig. 5), in oil, the chloroplast is going to the front rather than to the rear (Haupt and Scholz 1966).

Of course, immersion in oil has not only the physical effect of changing the collecting lens into a diverging one; moreover, some physico-chemical properties could have been changed. Therefore, we repeated this experiment with some synthetic oils of different refractive indices. As can be seen from Fig. 5, the percentage of chloroplasts going to the front, compared with those going to the rear, increases linearly with the refractive index of the oil, and no significant difference has been found between water and oil of the same refractive index. This is a very good proof for the lens effect being the mechanism of perception of light direction in *Hormidium*.

Before trying to answer the question of the nature and the localization of the photoreceptor, a complication has to be referred to. If light intensity is slightly increased, the type of orientation is changed fundamentally: all chloroplasts go to the front in air as well as in oil. This clearly demonstrates that now the lens effect is out of operation, and therefore we expect the attenuation effect to be working now. How can this be explained?

We put forward the hypothesis that orientation can be triggered by two different light reactions independently (Scholz and Haupt 1968): system A is operating with the lens effect, system B with the attenuation effect. How is it possible for both effects to work simultaneously in the cell?

Assume system A is localized in the cortical cytoplasm but system B in the chloro-

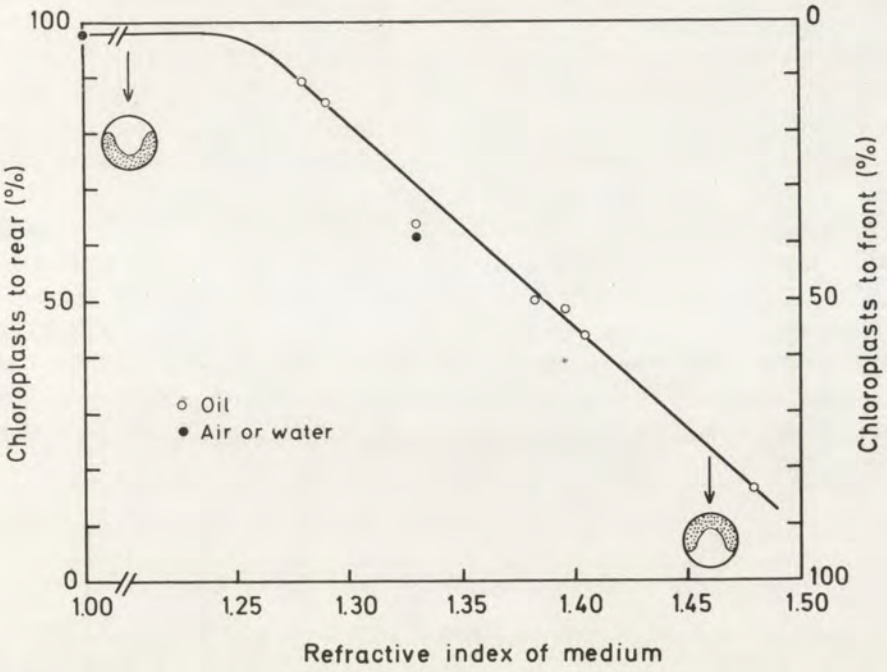

Fig. 5. Percentage of cells of *Hormidium* in which the chloroplast goes to the rear or to the front in response to unilateral blue light, depending upon the refractive index of the surrounding medium (circles = oil; dots = air or water, respectively). Type of response shown on upper left and lower right

plast. Obviously, in the chloroplast only attenuation of light occurs on its pathway. However, although light reaching the cortical cytoplasm at the rear has been partly attenuated, it has also been focused, and the latter might overcompensate the former.

To test this hypothesis, we had at least to try to separate both of these systems. This has been successful (Scholz in preparation):

(1) Though no true action spectra are available, we have found sensitivity of system A only in blue, that of system B in blue and, to some degree, in red light.

(2) Either system has an action dichroism, which, however, is quite different: in system A only blue light vibrating perpendicularly to the cell axis is effective (Fig. 4); in system B blue light has to vibrate parallel to the axis.

(3) Photoperception can be inhibited by potassium iodide in system A but not in system B.

(4) Dark culture for some days decreases the sensitivity of system A but not that of system B. Since the decreased sensitivity is restored by incubation with riboflavin, we may assume that a flavin is the photoreceptive pigment of system A. Nothing certain can be said concerning system B but it might be related to the photosynthetic apparatus.

To sum up, in *Hormidium*, light and light direction is perceived by two different systems, one being localized in the cortical cytoplasm like the system of Zurzycki in *Funaria*, the other being localized in the chloroplast itself. Though in air both systems act in opposite directions, viz. motion to the rear and to the front, respectively, this difference is of no importance for the plant: both situations enable the chloroplast to absorb maximum amount of light for photosynthesis.

As a second example I will refer to the filamentous green alga *Mougeotia* (cf. Haupt 1970 b). This alga is characterized by its single flat chloroplast (Pl. I 1 and 2) which turns according to light direction (Fig. 6). Again, we will be restricted to low intensity movement, i.e. turning from profile to face position to the light (Fig. 6 left). This response can be induced by very short irradiations and takes place, later, in complete darkness. As an exception, the photoreceptor in this alga is phytochrome, and by partial irradiations with the microbeam its localization at the cortical cytoplasm could be demonstrated, very probably at the plasmalemma.

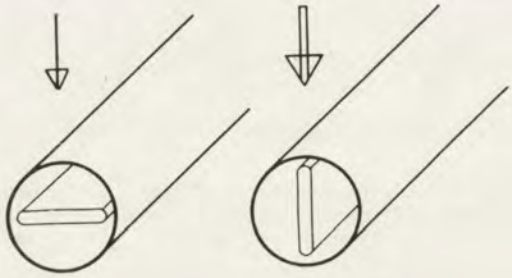

Fig. 6

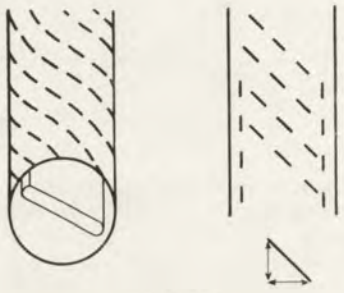

Fig. 7

Fig. 6. Cells of *Mougeotia* with a single flat chloroplast oriented in face and profile position to the direction of low and high intensity light, respectively

Fig. 7. Dichroic orientation of phytochrome molecules at the cell surface of *Mougeotia* (left). The dashes show the direction of main absorption. On the right, a surface view (middle part) and an optical section (edges) are combined schematically; below, longitudinal and perpendicular components of the oblique absorption vector are shown

By total and partial irradiations with polarized light, furthermore, a strong dichroic orientation of the phytochrome molecules has been detected; their vector of main absorption is oriented parallel to the surface and obliquely to the cell axis (Fig. 7). This means that absorption vectors can be found parallel and perpendicular to the cell axis, both in a plane parallel to the surface. This pattern is the base of the mechanism of perception of light direction in these cells, as will be shown now.

Consider a cell which is irradiated with polarized light. If the electrical vector of this light vibrates parallel to the cell axis, it will be absorbed all around the cell without any important gradient. If, however, vibration is perpendicular to the axis, this light will be absorbed only at front and rear, but not at the flanks. Consequently, if we mix together both kinds of polarized light or if we use, equally well, unpolarized light, absorption at front and rear is double as much as at the flanks. Thus, we

understand how dichroic orientation of the photoreceptor molecules has to result in a front-and-rear-to-flank gradient.

If phytochrome is irradiated with red light, the P_{660} form is transformed into the P_{730} form. In the *Mougeotia* cell, therefore, after red light we have to find much more P_{730} at front and rear than at the flanks. The chloroplast always moves in that way as to avoid with its edges the region with the highest concentration of P_{730} (Fig. 8); this has been shown by partial irradiations with the microbeam and this

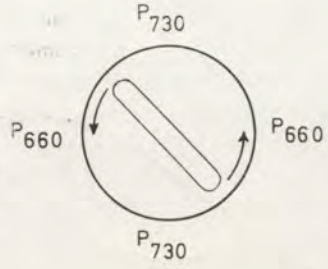

Fig. 8. Cross-section of a *Mougeotia* cell demonstrating the movement of the chloroplast in a gradient of phytochrome which is present mainly as P_{730} here and mainly as P_{660} there

also is the base of the normal response referred to here. At the moment we do not know the cause and the mechanism of this "avoidance-response". Instead, I have to call your attention to a further problem of establishing the absorption gradient.

It is a well known fact that phytochrome transformation from P_{660} to P_{730} needs very little energy. Even in not very high intensities, saturation is reached after a few minutes' irradiation. It is to be expected, then, that saturation will be reached at the flanks as well – in spite of less absorption observed there in comparison with front and rear. By this way, after saturating irradiation we expect that the gradient of P_{730} should be lost, we expect that high concentration of P_{730} should be established all around the cell. Nevertheless, even in continuous light orientation movement occurs, showing that the gradient is still strong. How can this be explained?

The answer to this question is given by details of the dichroic orientation of the pigment molecules. As has been stated above, the vector of main absorption of phytochrome is oriented parallel to the surface. This, however, is only true for the P_{660} form of phytochrome. The P_{730} form, in contrast, is oriented perpendicularly to the surface, i. e., in a radial direction (Fig. 9). This fact has been proven especially in the *Mougeotia* cell by proper irradiation with the microbeam (Haupt 1968, 1970 a). This has very important consequences for the photostationary state of the two forms of phytochrome. If phytochrome molecules are randomly distributed, saturating red irradiation results in 80% P_{730}, 20% remaining in the P_{660} form. If there is, however, dichroic orientation which is different for the two phytochrome forms, the photostationary state will be shifted depending on the geometry. This means, in the case of the *Mougeotia* cell (Fig. 10). At front and rear the dichroic orientation of P_{660} favours absorption, whereas that of P_{730} is unfavourable; the result will be a higher photostationary state of P_{730} in red, e. g. 90%. In contrast, at the flanks,

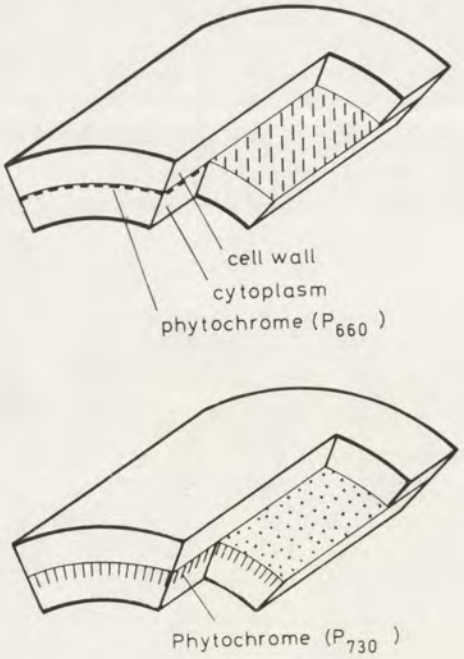

Fig. 9

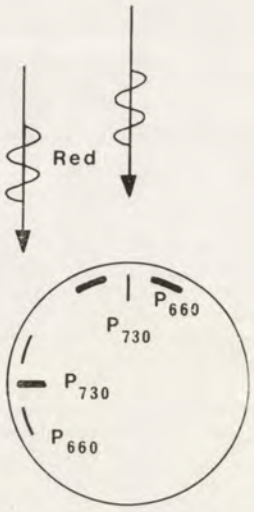

Fig. 10

Fig. 9. Part of cell wall and cytoplasm of *Mougeotia* given schematically, with the dichroic orientation of phytochrome molecules (cf. Fig. 7) in the P_{660} and in the P_{730} form, respectively
Fig. 10. *Mougeotia* cell (cross-section, without chloroplast) with phytochrome molecules (dashes, cf. Fig. 7 and 9) partly in the P_{660} and partly in the P_{730} form. Those molecules which are, according to their dichroic orientation, in the right position to absorb polarized light (arrows), are drawn by heavy lines

the dichroic orientation of P_{730} is more favourable for absorption (as far as the light vector vibrating perpendicularly to the cell axis is concerned) with the result of a lower photostationary state of P_{730}.

To sum up, saturation does not level out the gradient in *Mougeotia*, because at different sites different photostationary states will be established, due to the dichroic orientation of the phytochrome molecules which changes its direction when transformed from P_{660} to P_{730} and vice versa.

Nevertheless, there are problems in *Mougeotia* which are still unexplained at the moment. Without going into detail, some of them will be mentioned briefly.

(1) Besides red light, also blue light can induce the low intensity orientation movement, though with less efficiency. From recent investigations, this blue light effect is very likely not mediated by phytochrome. Thus, we would like to know the interaction of the blue light absorbing pigment and the phytochrome, both being localized in the cytoplasm rather than in the chloroplast proper (Haupt 1971).

(2) Whereas in this case light absorption in either of both pigment systems alone

results in the response, it is still more complicated in the high intensity orientation movement of the same alga: this latter response requires simultaneous absorption of light in phytochrome and a blue absorbing pigment. Nothing is known about the nature and localization of this blue absorbing pigment nor about its eventual identity with the blue light system of the low intensity movement (Haupt and Schönbohm 1970).

(3) Blue light of very high intensity forces the chloroplast of *Mougeotia* to reversibly contract seriously (Mörtel 1970). Nevertheless, even such contracted chloroplasts are able to orient to unilateral light (Weisenseel 1968). This, of course, is more interesting in view of the mechanism of movement that in view of perception of light direction.

In conclusion, even if a system of light perception seems to be adequately discovered as in *Mougeotia*, there eventually may arise new complications which make the response as a whole still more enigmatic, and much work has to be done in order to elucidate these complications.

Summary

Chloroplast movement is referred to as an example of light oriented displacement of cell organelles. Four possibilities are shown how the direction of unilateral light can be received by the cell. Besides these principal possibilities, two particular cases are referred to in more detail because of interesting complications:

(1) The alga *Hormidium* makes use of two different mechanisms of perception of light direction at the same time and concurrently.

(2) The alga *Mougeotia* avoids a saturation effect (which would result in levelling the absorption gradient) by changing the dichroic orientation of phytochrome between its two forms P_{660} and P_{730}.

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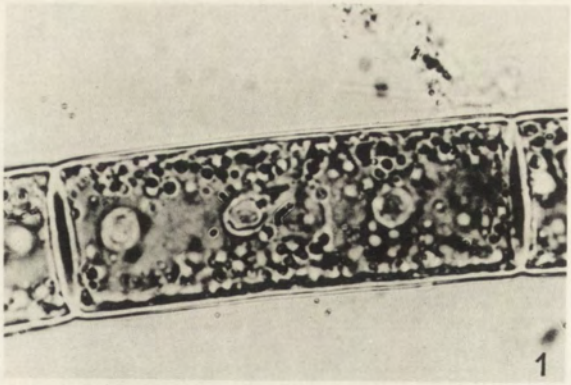
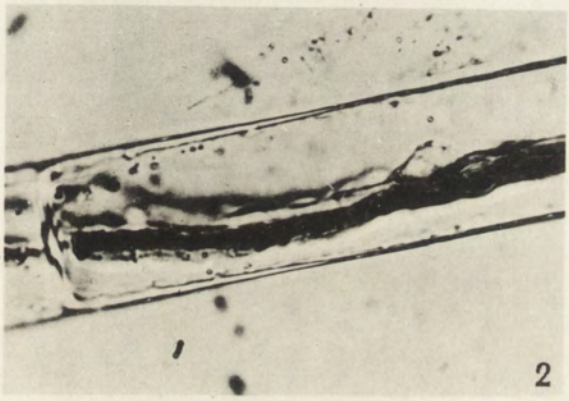
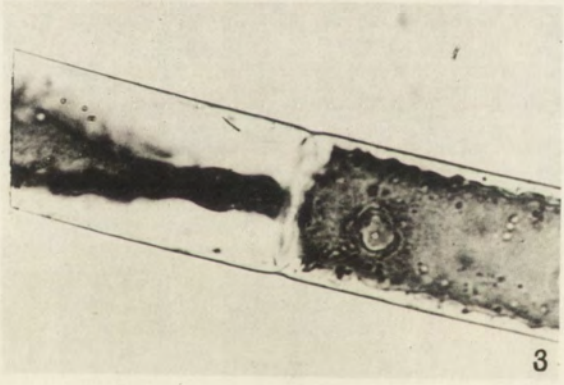
EXPLANATION OF PLATE I

1–3: Photographs of *Mougeotia* cells with the chloroplast in plane (1), profile position (2), and chloroplast position different in two neighboring cells (3)







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Primary reactions in the chloroplast rearrangements

It is well known that the chloroplasts of many plants do not have a stable position in the cell, but can displace themselves in the layer of cytoplasm and take different arrangements. The main factor controlling the position of chloroplasts is light, its intensity, wave-length and direction (Senn 1908).

Let us consider the plant cell illuminated from one side. In low light intensity the chloroplasts move to the cell walls perpendicular to the light rays and remain in that position. Looking at the cell from the direction of light one can see the chloroplasts in flat position (Pl. I 1 a). In strong light the chloroplasts displace to the side walls leaving the former walls free and take the so-called profile position (Pl. I 1 b).

If light exerts some influence on the position of chloroplasts, one must expect the existence of a pigment having the ability of light absorption and acting as a link between light energy and a chain of physiological processes leading to the end effect, i. e. the new arrangement of chloroplasts.

In low light intensity light absorption in this pigment at the best illuminated walls (i. e. situated perpendicular to the light direction) is great enough to ensure the most favourable conditions for chloroplasts. In high light intensity the absorption at these places becomes too great and on the less illuminated side walls it is big enough now to provide better conditions for them. When only a half of a cell is illuminated with strong light, typical strong light rearrangements take place in the illuminated part alone (Pl. I 1 c). In extremely high light intensities even on the side walls the absorption becomes too big and the chloroplasts come together forming a kind of clusters (Pl. I 1 d). It seems that the illumination changes the local conditions for chloroplasts in the cell.

The sequence of events taking place in the process of light-induced chloroplast rearrangements may be drawn schematically as follows:

$$A \rightarrow A^* \rightarrow P \rightarrow S \rightarrow LC \rightarrow T \rightarrow NP$$

Light is absorbed in the photoreceptor molecule (A). The excited photoreceptor (A*) controls the primary reactions (P), these in turn induce secondary reactions (S) changing the local conditions for chloroplasts (LC). If these conditions are not

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suitable there follows the next step – transport of chloroplasts to more suitable places (T) and taking a new position (NP).

This article is devoted to the photoreceptor problem and the primary reactions only. The problems of chloroplast transport will not be discussed.

At first it is necessary to point out that the position of chloroplasts, at least in some objects, can be evaluated quantitatively. This is possible by counting all the chloroplasts in some cells (for instance in the cells adapted to weak light when all chloroplasts are in flat position on the upper and lower cell walls) and then estimating the per cent of chloroplasts which remain in flat position under given light conditions (Zurzycki 1962). This per cent is 100 for low light intensity, 0 for high light intensity and takes intermediary values for medium or extremely low light intensities. In this way the determination of the light intensity curve of chloroplast arrangement is possible. Fig. 1 shows such curves for *Lemna* and *Funaria* cells taken in blue light 454 nm. These curves show, among others, that extremely weak radiation of the order of 1 erg or even a fraction of erg cm^{-2} sec^{-1} exerts just some influence on the position of chloroplasts.

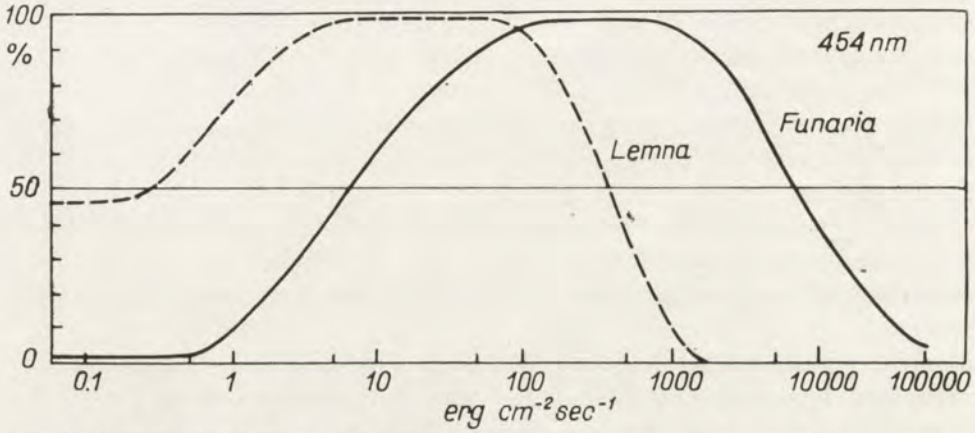

Fig. 1. Dependence of the arrangement of chloroplasts (Y axis —per cent of chloroplasts in flat position) on the intensity of blue light 454 nm. (X axis—light intensity in erg $cm^{-2}sec^{-1}$)

From such curves obtained for different wave-lengths one can estimate the action spectrum of light taking as an arbitrary criterion of activity, for instance, the intensity of radiation which induces 50% of chloroplasts to take flat position or remain in flat position.

The action spectra reported by Haupt and Schönfeld (1962) and Fischer-Arnold (1963) for *Vaucheria*, by us (Zurzycki 1962) for *Lemna*, by Mayer (1964) for *Selaginella* and some others show two peaks of activity at about 450 and 370 nm and no activity in the long wave range of the spectrum. The action spectrum for leaves of typical land plants reported at this conference by Dr Lechowski has essentially the same character. Some years ago we succeeded to measure the action spectrum

for *Funaria* leaves by extending the study into UV region and to show the main peak of activity at 266 nm (Fig. 2). The shape of the obtained action spectra in visible range as well as in UV shows great similarity to the absorption spectrum of riboflavin solution. But no decisive conclusion about the nature of the photoreceptor can be drawn from this agreement.

More information concerning the photoreceptor active in the chloroplast rearrangements can be found in the reaction of chloroplasts to polarized light. Linearly polarized blue light induces the rearrangements strictly dependent on the direction of polarization. In strong light the chloroplasts move as usual to the side walls but avoid the walls situated parallel to the electrical vector of light (Mayer 1964, Zurzycki 1967 b) (Pl. II 2).

A characteristic response of chloroplasts to polarized light may be explained by the

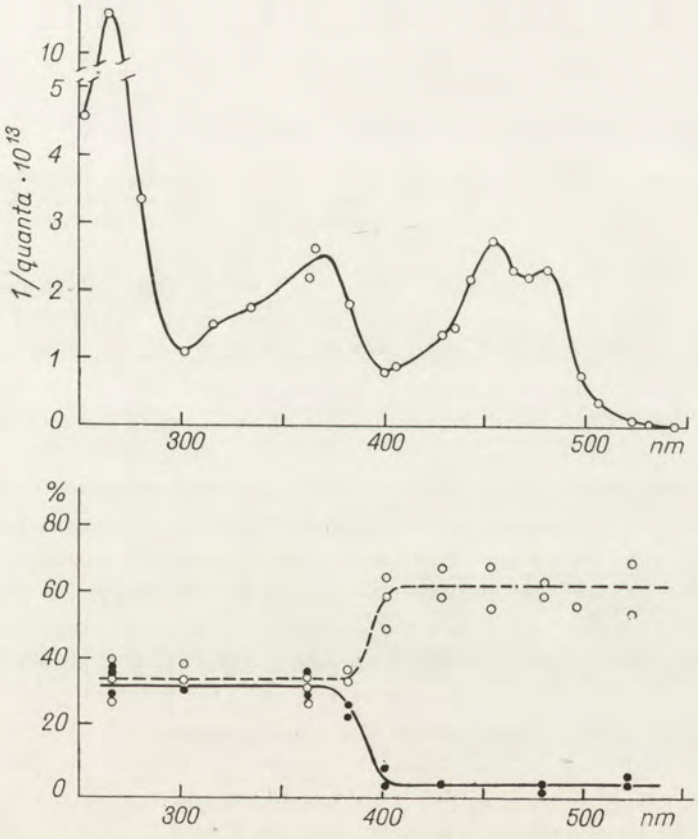

Fig. 2. Upper curve: action spectrum for chloroplast rearrangement in *Funaria* leaves. X axis—wave-length of radiation, y axis—reciprocal of the number of quanta inducing 50% of chloroplasts to take flat position (Zurzycki 1967 a). Lower curves: action dichroism in *Funaria* cells. X axis—wave-length, Y axis—measure of the occupation of a unit of length of the side walls by chloroplasts. – – o – – side walls perpendicular and —— • —— parallel to the E vector of polarized light (Zurzycki 1967 b)

supposition that the active photoreceptor is dichroic and that its molecules are in some way oriented in the cell. In this orientation the absorption vector is always oriented parallel to the cell walls. Looking for the cell structure which would show great enough stability and great enough orientation to be the site of the photoreceptor we come to a conclusion that the photoreceptor molecules are situated and oriented in the cell membrane complex (Zurzycki 1968) (Fig. 3).

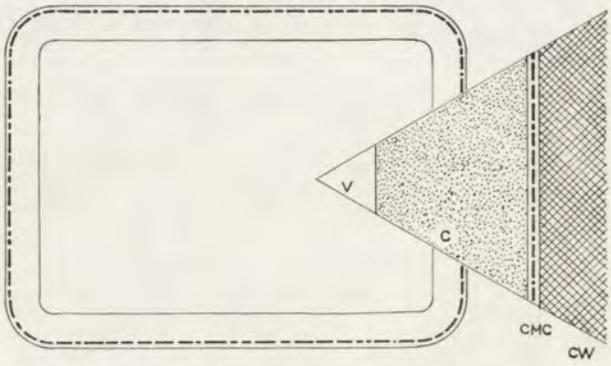

Fig. 3. Scheme of the orientation and localization of the phtoreceptor molecules in the cell. Thick lines represent the transition vector for blue light of the receptor molecules. V—vacuole, C—cytoplasm, CMC—cell membrane complex, CW—cell wall

The phenomenon of the action dichroism in chloroplast rearrangements takes place in blue light only. We found quite unexpectedly (Zurzycki 1967 b) that polarized UV exerts no directing influence on chloroplasts. The reaction to polarized UV is the same as to unpolarized one (Fig. 2).

Absorption spectrum of riboflavin consists of three peaks corresponding to the three transition moments of the molecules. As has been recently stated by Kurtin and Song (1968), the transition vectors are situated in the following way in a riboflavin molecule. The vector responsible for blue absorption peak is orientated nearly parallel to the longer axis of the isoalloxasine ring of riboflavin molecule. The vectors responsible for 370 and 260 nm peaks are situated at the angles 30–50° in respect to the longer axis of this ring. It means that when we assume the orientation of flavin molecules as shown in Fig. 4, the differences in absorption in blue are very significant between the side walls parallel and perpendicular to the plane of polarization, but the differences for UV absorption are very small or none, which agrees with the behaviour of chloroplasts. The agreement between the reaction of chloroplasts and optical properties of flavin molecule is a second argument speaking in favour of flavins (and not carotenoids) as an active photoreceptor.

The third argument may be drawn from the fact that extremely low intensities of radiation are active in some rearrangements. It seems that the effect of light is multiplied by probably an enzymatic activity. We know a lot of enzymes containing flavins, which is not the case with carotenoids.

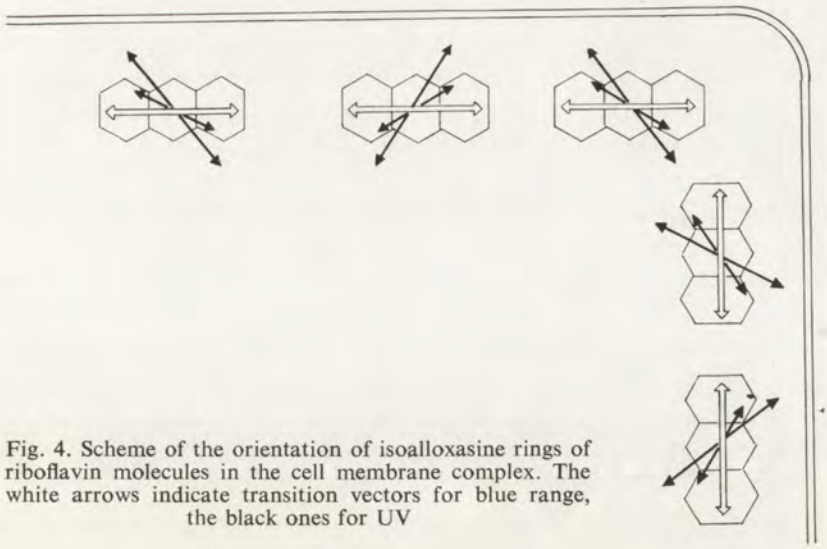

Fig. 4. Scheme of the orientation of isoalloxasine rings of riboflavin molecules in the cell membrane complex. The white arrows indicate transition vectors for blue range, the black ones for UV

Before looking for this enzyme let us consider another question. Are there any other physiological processes which are in a similar way dependent on light intensity, wave length and polarization? The data from the literature point to two such reactions: ability of chloroplasts for centrifugation and extra oxygen uptake (Table 1).

Table 1

	Light intensity	Wave-length	Polarization
Chloropast rearrangements	Senn 1908 Voerkel 1933 Zurzycki 1962	Haupt-Schonfeld 1962 Zurzycki 1962 Fischer-Arnold 1963	Mayer 1964 Zurzycki 1967 b
Ability for centrifugation	Virgin 1951	Virgin 1952 Virgin 1954	Seitz 1967
Extra oxygen uptake	Kowallik 1966–69 Zurzycki 1970	Kowallik 1967 Kowallik 1969	Zurzycki 1971

As was shown by Virgin (1951), in leaves adapted to weak light the chloroplasts are very resistant to centrifugation; after strong illumination the centrifugation is much easier. Virgin (1952, 1954) studied the action spectrum of this phenomenon and found the greatest spectral activity at 450 nm and no activity in red. More recently Seitz (1967) has succeeded to show that after illumination of *Vallisneria* cells with polarized light the chloroplasts situated at the side walls parallel to the E-vector of polarization are very easy to move during centrifugation.

Extra 0_2 uptake which takes place after illumination was found for *Chlorella* by Kowallik (1966, 1967, 1969) and also for *Lemna* and some mosses (Zurzycki

1970, 1971). Very low intensities of light are active in these cases and as we were able to find for *Lemna* leaves high intensities suppress the extra 0_2 uptake. Recently we have found that the effect of polarized light on leaves containing spindle-shaped, elongated cells is much greater when the E-vector of polarization is directed parallel to the cell axis than when it is perpendicular to it (Zurzycki 1971).

The three mentioned phenomena: rearrangements of chloroplasts, ability of chloroplasts for centrifugation and extra oxygen uptake seem to be controlled by the same photoreceptor and probably the same primary enzyme.

There are many enzymes containing FMN or FAD and the activity of some of them depends on light. According to the recent systematic studies of Schmid (1970), in most cases illumination suppresses their activity. Schmid and Schwarze (1969) found one FMN-mediated enzyme, which is not active in darkness but only in blue light. This is desaminating glycine oxidase. The authors explain the extra 0_2 uptake in *Chlorella* after illumination to be a result of the effect of light on this particular enzyme.

Combining all these facts I should like to propose a working hypothesis – which should explain the primary photoreactions in the light – induced chloroplast rearrangements.

The key enzyme – glycine oxidase – is situated in the cell membrane complex in such a way that isoalloxasine ring of the flavin component is oriented in respect to the cell surface, its plane perpendicular and its longer axis parallel to the surface.

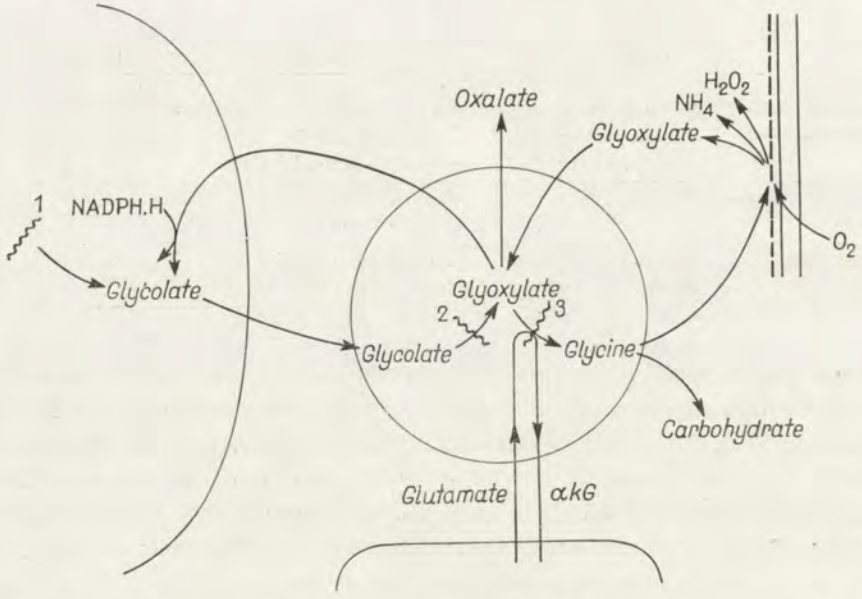

Fig. 5. The glycolate pathway and glycine-glyoxylate cycle in plant cell. Inhibitors: 1–DCMU, 2–α hydroxysulfonate, 3–aminooxyacetate

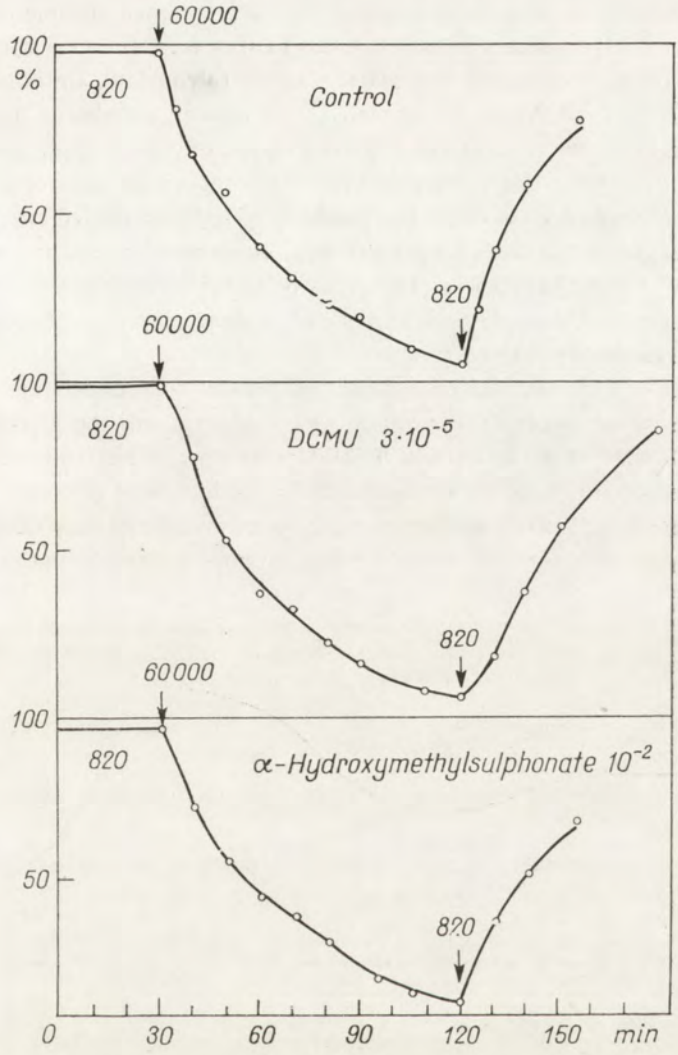

Fig. 6. Time course of chloroplast rearrangement in *Funaria hygrometrica*. X axis—time in minutes, Y axis—per cent of chloroplasts in flat position. Figures by the curves indicate the intensity of blue light (454 nm) in erg cm^{-2} sec^{-1}. Upper curve—control, middle curve DCMU 3×10^{-5} M/l, lower curve—α-hydroxymethylsulfonate 10^{-2} M/l

Light is a trigger factor for this enzyme, which is inactive in darkness and rather sensitive to light. The substrate for this enzyme – glycine – is formed in a glycolate pathway. Glycolic acid is produced as a by-product of the Calvin cycle in chloroplasts and oxidased to glyoxylate in the glyoxysomes, where also a change to glycine mediated by glutamate-glyoxylate transaminase takes place (Tolbert and Yamazaki 1969). Glycine leaves glyoxysomes and may be directed through serine and gly-

cerate to carbohydrates or may be oxidased by the mentioned enzyme to glyoxylate which enters the glyoxysomes again (Fig. 5). In this way we get a cyclic process. One or some of the products of enzymatic reaction (glyoxylate, ammonium ions or hydrogen peroxide), which are accumulated near the cell membrane during enzymatic reaction in higher concentration are the reason of changing the conditions for chloroplasts. In other words, the problem of phototaxis of chloroplasts may be explained by chemotactic action of the products of light-controlled enzymatic reaction. Illumination of the cell changes the local concentration and the gradients of concentration of these substances. The areas of suitable concentration become the attraction centre for chloroplasts, which move to these places and become in some way anchored in these positions.

What kind of experimental results can we present to support this hypothesis?

(1) Action of inhibitors. Three inhibitors interfering with the glycolic pathway were used. DCMU (Fig. 5, *1*), which inhibits non-cyclic electron transport and consequently slows down the Calvin cycle and the production of glycolate, α-hydroxymethylsulfonate (Fig. 5, *2*) a competitive inhibitor of glycolic oxidase (Zelitch 1957) and aminooxyacetate (AOA) – a well-known inhibitor of transaminases (Fig. 5, *3*).

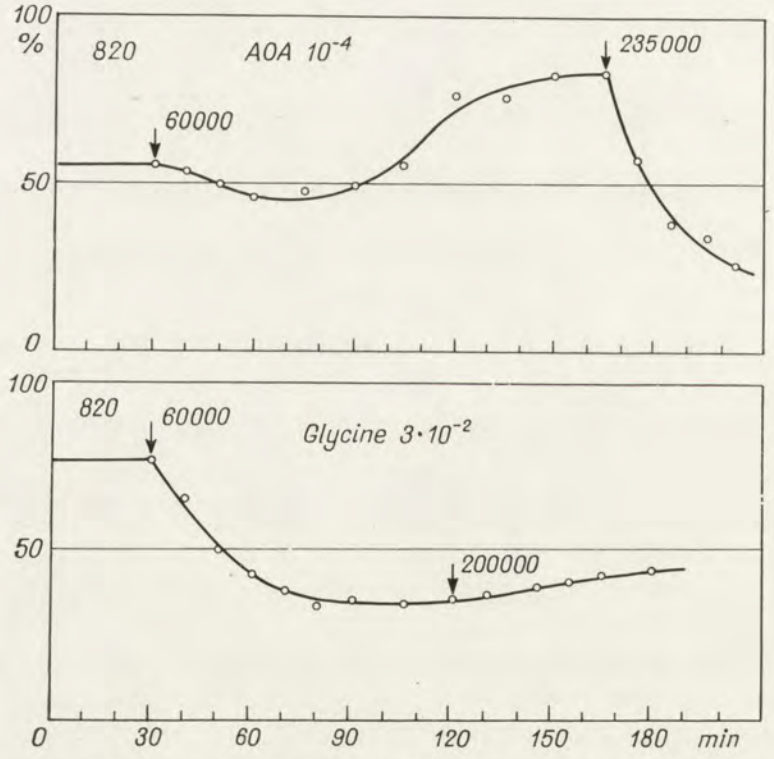

Fig. 7 Time course of chloroplast rearrangement. Upper curve – AOA 10^{-4} M/l, lower curve – glycine 10^{-2} M/l, Details as in Fig. 8

Figure 6 shows the displacements of chloroplasts in time by using of the first two inhibitors. Strong light rearrangement of chloroplasts as well as the reverse displacements are normal as in control leaves. On the other hand in AOA no complete low light intensity position was obtained in weak light (Fig. 7). In high light intensity which formerly evoked strong light position a reverse situation, i.e. movement of chloroplasts to low light position can be seen. Movement to strong light position is only possible by using a very high light intensity of about 250 000 erg cm^{-2} sec^{-1}. As a result of a comparison of these results with scheme 7 a conclusion may be drawn that for controlling of chloroplast position the recycling of the inner pool of glycine and glyoxylate is fundamental rather than the production de novo of the substrate. Fig. 8 shows the light intensity curves of chloroplast position. It shows clearly the decreasing of the sensitivity to light by using of A0A and reverse, the increase of the sensitivity by addition of glycine.

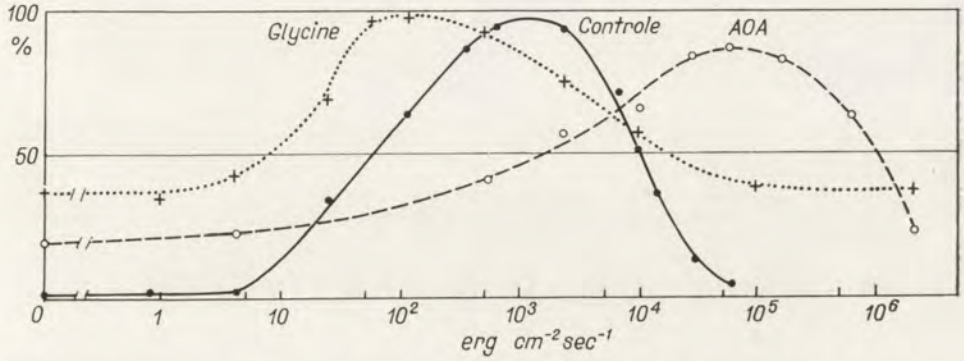

Fig. 8. Light intensity curves of chloroplast arrangement for control material and for leaves treated 2–3 h in glycine 10^{-3} or AOA 10^{-4} M/l solutions

(2) The activity of the enzyme responsible for extra oxygen uptake is found in *Lemna* leaves in vivo like in Schmid's experiment performed in vitro to depend on illumination. The oxygen uptake by dark-adapted leaves in different light intensities and at different concentration of a substrate (in this case glycolic acid) is presented in Fig. 9. In the dark and very low light intensities the enzyme is independent of a substrate concentration. The influence of external glycolate concentration is visible only in higher intensities of light, i.e. after activation of the photoreceptor. Similar results may be obtained with glycine as a substrate.

(3) It was found at least that A0A really suppresses the extra oxygen uptake (Table 2).

From our hypothesis a supposition may be drawn that one or some of the products of desaminating glycine oxidase would act by local application chemotactically on chloroplasts.

Table 2

Oxygen uptake in blue light 454 nm, 8 erg cm^{-2} sec^{-1} intensity in per cent of dark respiration. *Lemna trisulca*

Control	130.6
10^{-6} AOA	126
10^{-5} AOA	120
3×10^{-5}	103
10^{-4} AOA	100.4

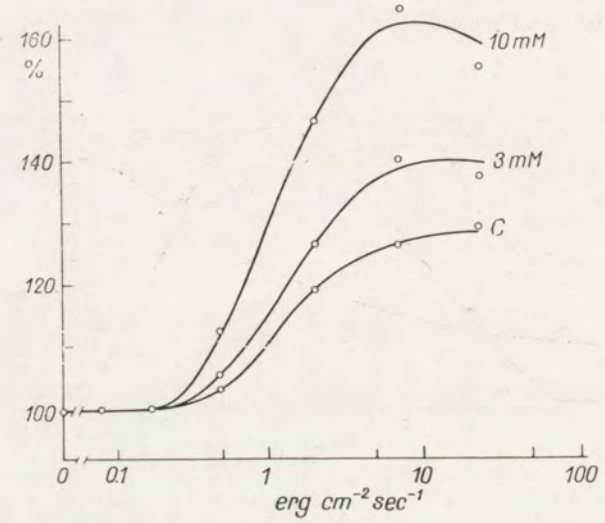

Fig. 9. Dependence of extra O_2 uptake (Y axis—oxygen uptake in per cent of dark respiration) on light intensity (X axis—intensity of 454 nm light in erg cm^{-2} sec^{-1}) in *Lemna* leaves, for control (medium—water) and 3 or 10×10^{-3} M/l glycolic acid adjusted to pH 5.3 by KOH

Summary

The reactions of chloroplasts to light intensity, wave-length and direction of polarization were discussed. On the basis of a similarity between these reactions and a response of chloroplasts to centrifugation as well as light dependent extra O_2 uptake, the conclusion was drawn that all three reactions are controlled by the same photoreceptor. A working hypothesis was postulated concerning the primary reactions of chloroplast rearrangements. According to it light is a trigger factor for FMN-mediated enzyme (glycine oxidase), localized and oriented in the cell membrane complex. The concentration of reaction products of the desaminating glycine oxidation is essential for creating the local conditions for chloroplasts.

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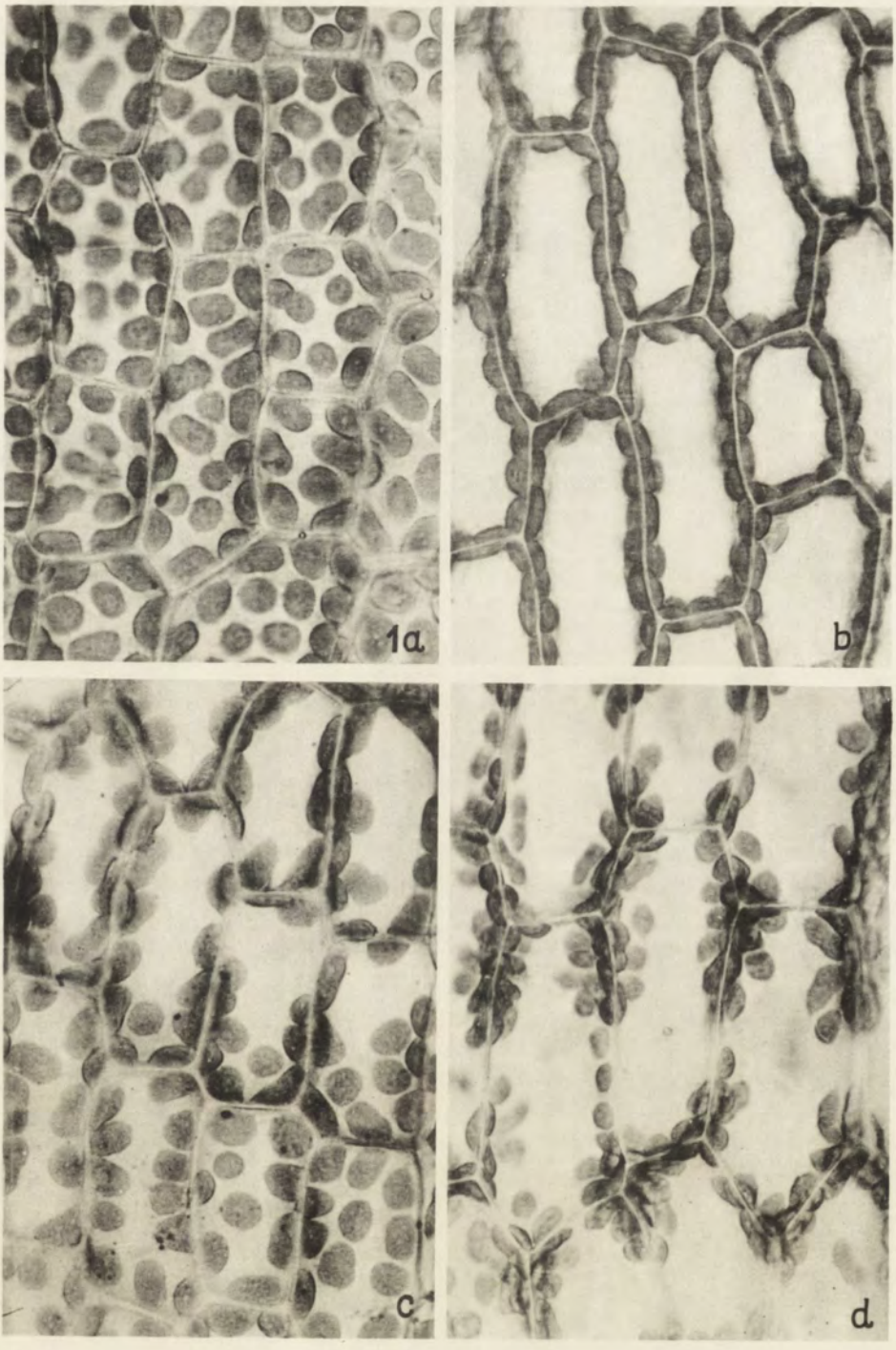
EXPLANATION OF PLATES I–II

1: Arrangements of chloroplasts in the leaf cells of *Funaria hygrometrica*. a — flat positioon typical for low light intensity, b — profile position of chloroplasts, c — strong light position iin the upper part of the picture and low light position in the lower part, d — clusters of chloroo-plasts induced by very strong irradiation

2: Chloroplast arrangement in *Funaria* cells after irradiation with strong linear polarized bluue light. The E vector directed parallel to the shorter side walls



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Action spectrum of chloroplast displacements in the leaves of land plants

The chloroplast displacements take place in the cells of numerous plants and they depend mainly on various intensities of illumination. The dynamics of chloroplast movements was studied in various species of plants whose leaves or other vegetative organs are built of 1–3 layers of cells. A microscopic observation and determination of the number of chloroplasts that change their position became the base for a quantitative evaluation for this group of plants. It is impossible to apply this method to a multilayer leaf. It has often been noticed that the optical properties of the leaf and first of all the transmission and reflection ability change in accordance with the light conditions acting upon the leaf. It has also been found that these changes are closely correlated with chloroplast system in the leaf cells. The profile position in the chloroplast arising under the influence of high light intensity reduces the amount of absorbed light energy and the amount of transmitted light increases.

The influence of different light conditions upon this process was investigated with the method of light transmission measurements.

Schematic graph shows the apparatus for the measurement of the transmission (Fig. 1). A microscopic lamp fed by a stabilizer and autotransformer was the source of light. The light beam from the lamp went through a suitable interference filter. The investigated leaf was put on a moveable opal plate and covered with a piece of glass in order to be fixed in a flat position.

The leaf surface subject to measurements was limited to 8×2 mm which made it possible to carry out the measurements on a part of the leaf without bigger nerves. A neutral filter of well-known transmittance that could be shifted deffected away from the light beam was placed under the opal plate. This filter could be easily eliminated. Photomultiplier was used as a light detector. The intensity of incident light (opalescent glass) was always measured with a neutral filter applied.

The plate with the leaf was subsequently shifted. Measurement of the light transmitted through the leaf (opalescent glass + leaf) was performed with the neutral filter removed. Such a measurement system ensured readings with an accuracy up to

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

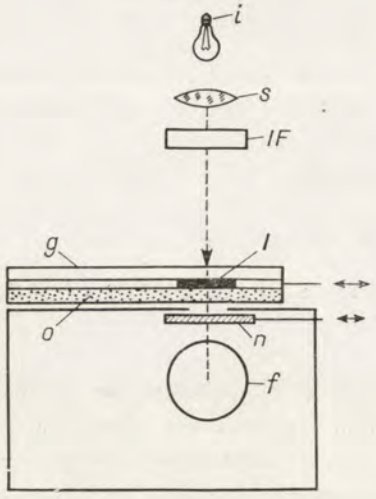

Fig. 1. Diagram of the apparatus for light measurements i—microscopic lamp, s—lens, IF—interference filter, g—normal glass, l—leaf, o—opalescent glass, n—neutral filter, f—photomultiplier

0.06% of transmittance. The transmission values were given in percent or in the form of semi-integral attenuance.

After 1 h of leaf exposure to the white light of the colour temperature of 6000°K the spectral changes of transmission for the series of plant species were established.

Figure 2 shows spectral changes in transmission for some species of plants after irradiation with strong light intensity. In the species reacting most intensely, e. g., *Ajuga reptans* (Fig. 2 a), distinct spectral changes are present in the short wave-length part of spectrum below 481 nm and also in the range of 597–680 nm. The range of 481–597 and over 680 nm gives insignificant changes of transmittance. *Galeopsis tetrahit* shows a similar degree of reaction (Fig. 2 b). *Vitis vinifera* (Fig. 2 c) and *Fagus silvatica* (Fig. 2 d) belong to the second group of much weaker reacting plants. The transmission increase in this group of plants is almost identical in the whole visible range. Such species as *Rhus typhina* (Fig. 2 e) and *Vicia faba* (Fig. 2 F) show only an insignificant increase of transmission.

The exemplar changes in transmission measured for $\lambda = 436$ nm result for:

Ajuga reptans from 1.5% of transmittance before exposing to high light intensity to 6.93% after irradiation,

Vitis vinifera – 1.92–2.82%,

Rhus typhina – 0.30–0.46%.

It is evident from the data shown in diagrams the transmission increase in the ranges of strong light absorption is mainly due to photosynthetic pigments.

The transmission increase is connected with the decrease of light absorption. The transmission increase for the whole visible range as calculated planimetrically is: for

Ajuga reptans – 4.9%,

Galeopsis tetrahit – 3.1%.

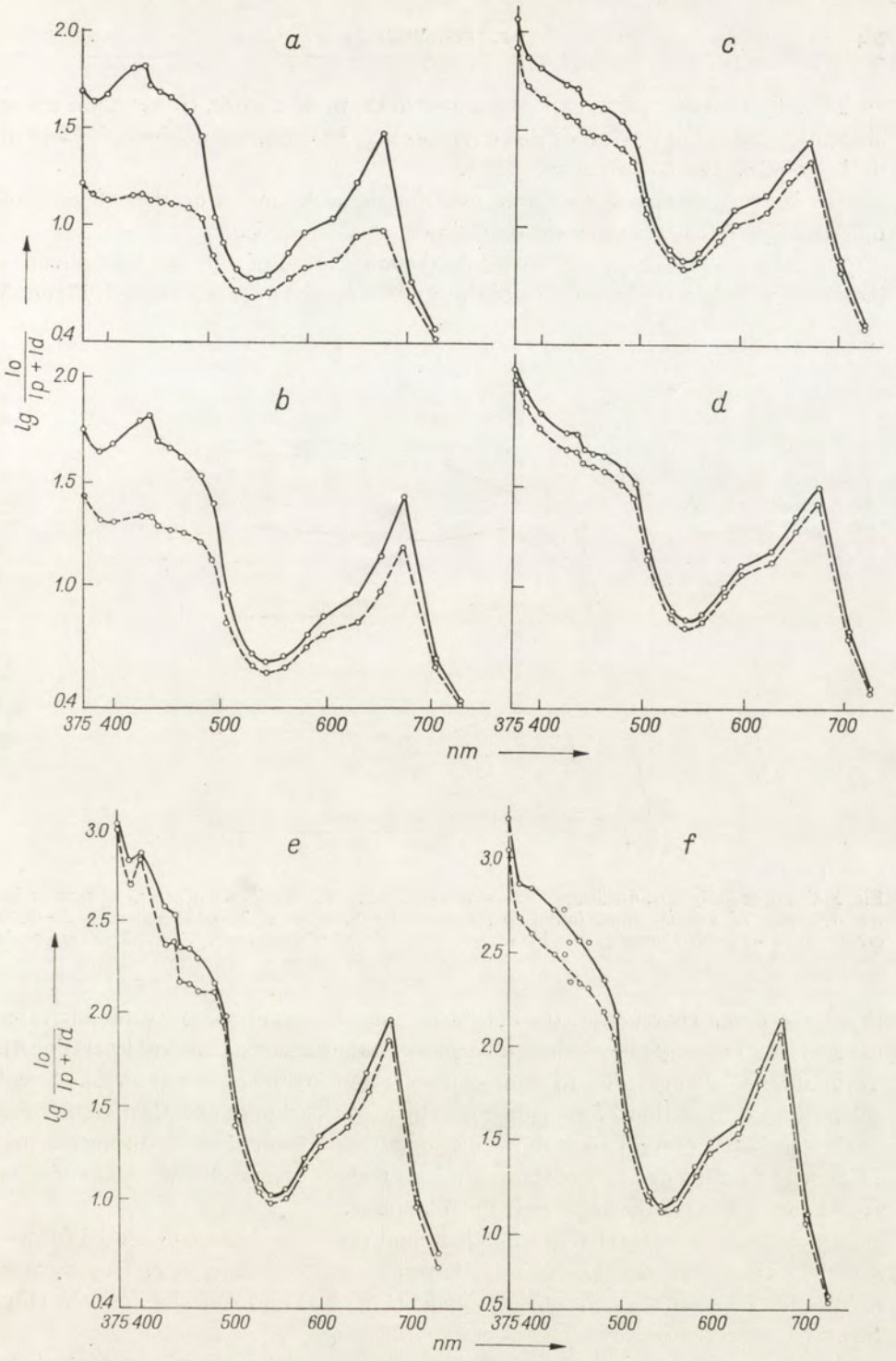

Fig. 2. Spectral changes in transmittance after 1 h of exposure to white light of 131 thousand erg/cm² × s. ○———○ — leaf transmittance in weak light, - - ○ - - ○ — leaf transmittance after irradiation, a—*Ajuga reptans*, b—*Galeopsis tetrahit*, c—*Vitis vinifera*, d—*Fagus silvatica*, e—*Rhus typhina*, f—*Vicia faba*

The reflection changes were not measured in the present work. However the entire absorption change in the leaf of *Ajuga reptans* may be estimated at about 6% and in the leaf of *Galeopsis tetrahit* at about 5%.

The leaves of *Ajuga reptans* were used for the experiments on the influence of different light intensities upon the optical properties of the leaf.

The transmission changes recorded on the application of different light intensity indicate a pronounced dependence of this process on the intensity applied. Figure 3

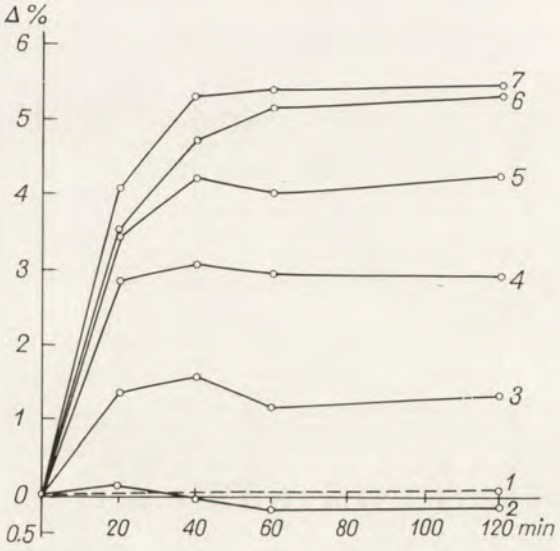

Fig. 3. Changes in light transmittance of 436 nm wave-length in the leaves of *Ajuga reptans* under the influence of various light intensities, curves: 1—0 erg/cm²s, 2—4000 erg/cm²s, 3—9000 erg/cm²s, 4—18 600 erg/cm²s, 5—36 000 erg/cm²s, 6—65 000 erg/cm²s, 7—253 000 erg/cm²s

shows the data. They permit the conclusion that the transmission value increases to some light intensity above which it becomes established on a constant level (Fig. 4). Transmittance changes due to strong illumination are reversible (Fig. 5). A leaf submitted to the action of very intensive light for six hours and then transferred to the conditions of weak light shows a strong transmission decrease during the first 30 min. The further decrease of transmittance proceeds at a much lower rate and after four hours it reaches the value near the initial one.

The exposition performed in white light and the transmittance measured for $\lambda =$ 436 nm, $\lambda = 506$ nm and $\lambda = 673$ nm have identical course in time. This reaction follows the identical way for *Ajuga reptans* (Fig. 5 a) and *Galeopsis tetrahit* (Fig. 5 b).

It is stated on the basis of numerous studies on the influence of part of the light spectrum on transmission changes that the short wave-length is acting in this pro-

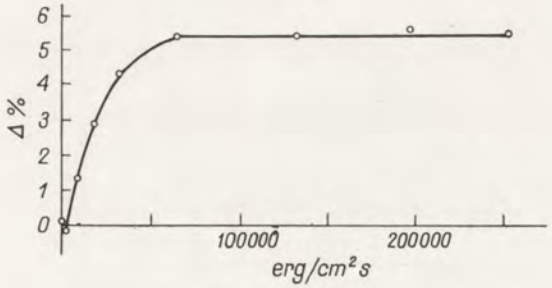

Fig. 4. Percent changes of transmittance in the leaf of *Ajuga reptans* measured at $\lambda = 436$ nm when stabilization was reached after irradiation with white light of varying intensity (x axis)

cess while the long wave range does not produce any greater effect. However, till now no exact determination of the action spectrum for the mentioned process of the monochromatic light influence on the leaves of land plants has been reported.

The present experiments were carried out on the leaves of *Ajuga reptans*. The illuminations were performed in a special apparatus for irradiation. The Fig. 6 shows the apparatus for irradiation. The projector lamp of 110 V 750 W was the source of light. The light was concentrated by means of an optical system which gave an evenly illuminated spot of 2 cm in diameter. A water filter and an interference filter were inserted into the optical system. The light measurement was performed with a Zeiss thermopile VTh 8 EN. The light-proof chamber was surrounded with a water jacket to maintain a constant temperature of 25°C. After 2 h of the leaf irradiation

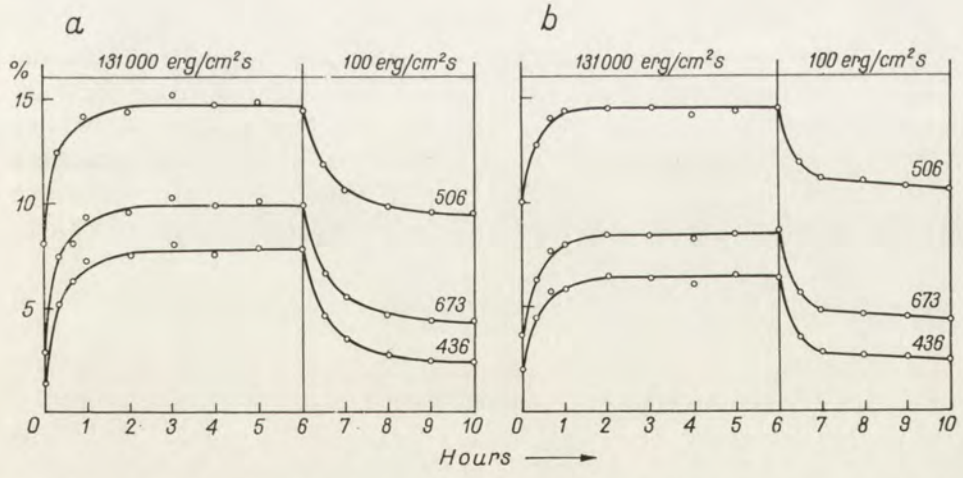

Fig. 5. Changes in transmittance during irradiation with white light of 131 thousand erg/cm²s intensity measured at 436, 506 and 673 nm

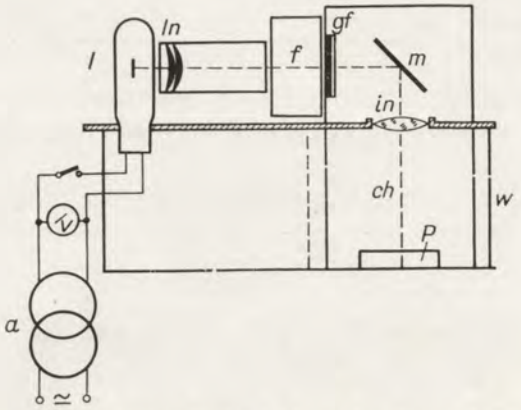

Fig. 6. Scheme of the apparatus for irradiation l—lamp, ln—lenses, f—filter with $CuSO_4$ solution, gf—glass filter, m—mirror, ch—chamber for illumination, P—Petri dish, v—volta-meter, a—autotransformer, w—water jacket

with monochromatic light the arisen transmittance changes were examined for $\lambda =$ $= 436$ nm. Figure 7 shows the obtained curve system.

The light whose wave-length is above 502 nm does not exert any influence upon the transmission increase. The obtained transmission values, e.g. for $\lambda = 628$ nm, in spite of increasing light intensities remain at a constant level. The light intensity inducing 50% of reaction was adopted as a calculation criterion. It makes amounts to $\Delta 3\%$. The light intensity values in thousands of erg/cm² s at which the same reaction level was obtained were converted to quanta number. Figure 8 presents the results. Curve I shows the diagram of the obtained action spectrum. Two ranges of action activity can be distinguished on the curve. The first one with the maximum in the near UV for $\lambda = 375$ nm and the other at 481 nm.

The light acting upon the leaf is to a considerable extent absorbed by it. This is why it seems necessary to take into consideration the calculation correction for autoabsorption. The correction was calculated by means of Lambert–Beer law, taking the thickness of absorption layer equal to 1/2, 1/3, 1/5 and accepting that the leaf is a homogenic medium. The absorption coefficient was calculated for each wavelength on the basis of light amount absorbed by the leaf adapted to weak light conditions. The data are shown on Fig. 8.

Assuming that the thickness of the absorption layer is 1/5, one obtains the curve close to the previous one (Curve II). There is the maximum for $\lambda = 375$ nm and $\lambda = 481$ nm distinguishable in the action spectrum for *Ajuga reptans*. The next diagram presents the specification of action spectrum obtained for different objects (Fig. 9).

It is difficult to qualify precisely the action spectrum in the leaves of land plants because of a complex structure of the leaf. However, the obtained results suggest the existence of a similar photoreceptor of the flavin group of pigments, such as has

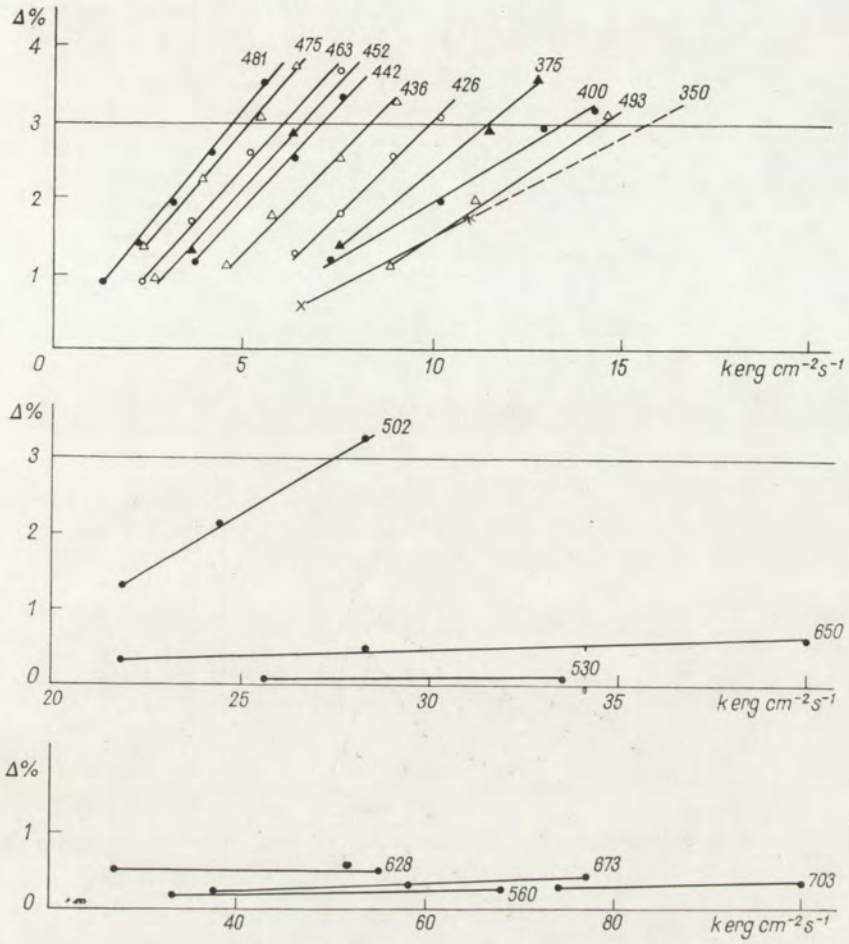

Fig. 7. Effect of transmission changes obtained under the influence of 2 h irradiation with monochromatic light. (The wave-length is designated with numbers over the curve)

been obtained for simple objects. This is indicated by the maximum present in near UV and the maximum in the blue light which for *Ajuga reptans* is reached at 481 nm. The reason for the shift of the latter maximum is rather difficult to explain. Up to now only Babuškin 1955 has obtained the action spectrum for multilayer leaves of *Nicotiana tabacum*. Nevertheless his method of contact prints on printing-paper seems to be insufficient to study the action spectrum in the displacement of the chloroplasts in leaves. Our results obtained for *Ajuga reptans* show no correlation with his findings.

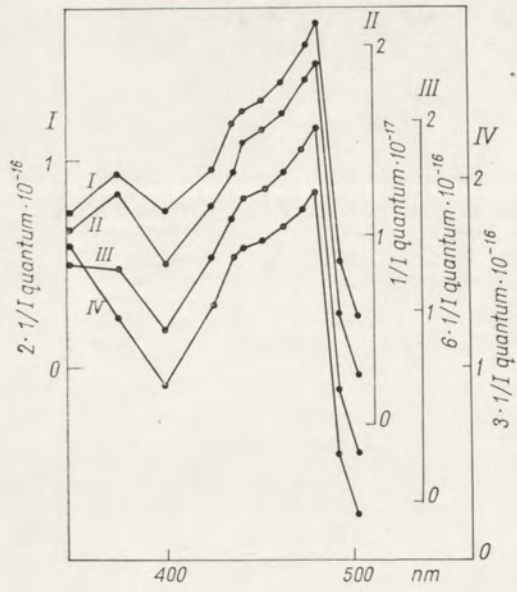

Fig. 8. Action spectrum in chloroplast displacements of *Ajuga reptans*: I – not corrected, II, III, IV – corrected for the autoabsorption of the leaf for 1/5, 1/3 and 1/2 of its thicken ess respectively

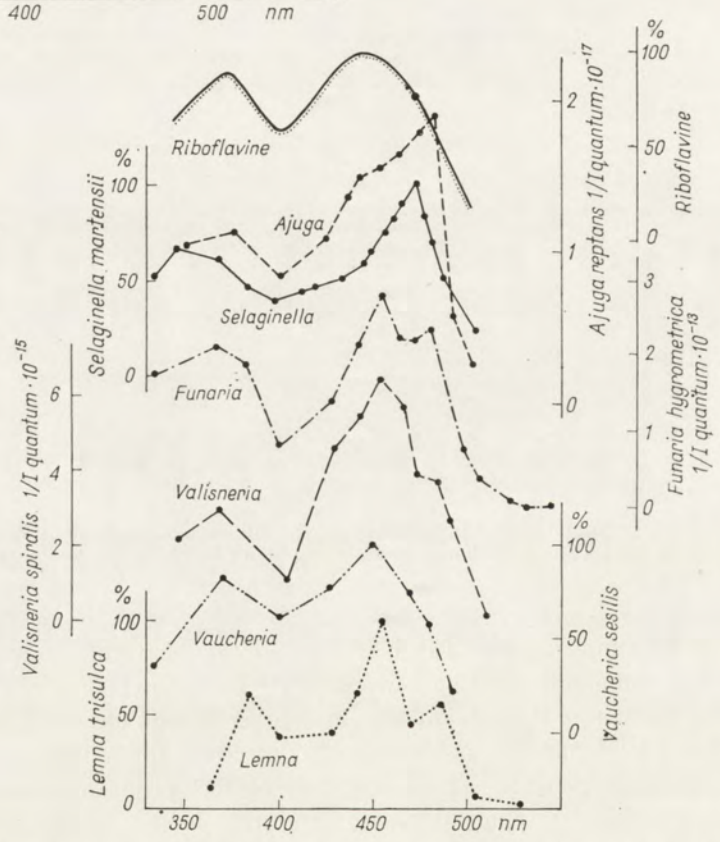

Fig. 9. Action spectrum in chloroplast displacements in the cells (*Lemna*: Zurzycki, 1962; *Vaucheria*: Haupt and Schonfeld, 1962, Fischer-Arnold, 1963; *Selaginella*: Mayer, 1964; *Vallisneria*: Seitz, 1967; *Funaria*: Zurzycki, 1967; *Ajuga*: Lechowski) and riboflavin absorption

Acknowledgement

I am deeply indebted to Professor J. Zurzycki, Head of the Department of Plant Physiology for his valuable advice at my present work.

Summary

The investigations upon the changes in transmittance of radiation through a leaf caused by various light intensities acting on the leaves under the controlled conditions were discussed in the present paper. Evidence was given for the existence of 3 plant groups showing different levels of chloroplast reaction for the acting light stimulus. The transmission changes were found to depend on the light wave-length, as well as on the intensity and the acting time of light stimulus. The investigations on the action of the spectrum on changes in transmittance point to the existence of 2 maxima for $\lambda = 481$ nm and $\lambda = 375$ nm. Such a diagram of maxima suggests the existence of the riboflavin pigment group in this process.

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Experiments on the mechanism of chloroplast movement in light oriented chloroplast arrangement

In 1962 a very interesting article about the problem of the mechanism of chloroplast movement was written by J. Zurzycki, published in Ruhlands Handbook of Plant Physiology 17/2. This review presents summarized results of former experiments on this problem. Only few results have been obtained since then (see: Haupt and Schönbohm 1970). There is not enough time here to give a satisfactory report either on the former results in general or on all formulated hypotheses which are known. Let us go now in medias res.

First I will give you a short disposition of this paper (items 1–6), then we have to look at the results of our experiments.

(1) In nearly all investigations we experimented with the filamentous green alga *Mougeotia* sp. Only in few experiments did we test some effects on the moss *Funaria hygrometrica*.

(2) I will give you a short survey of some results of former experiments on the photoreceptor and energetic problems of light-oriented chloroplast movement of *Mougeotia*.

(3) An attempt should be made to formulate my hypothesis on the mechanism of chloroplast movement.

(4) We have to look at the effect of SH-blockers on light-oriented chloroplast movement.

(5) What is the effect of external factors as light and dark and SH-blockers on fastening of chloroplasts to cytoplasmic parietal coating?

(6) Can we see any relation between cytoplasmic structures and a possible function of these structures in the mechanism of chloroplast movement?

(1) The objects: In Plate I you can see on the top (A) a survey of an unpretreated preparate of *Mougeotia*, showing chloroplasts in different positions. At higher magnification (B) you can see two single *Mougeotia* cells, each with only one large chloroplast (chl), the one in plane and the other in profile position. At higher magnifications (C, D) further details can be observed: pyrenoids (p), plasmic filaments (f), plasmic parietal coating of the cell wall (ppc) and a relatively large nucleus (n) laying

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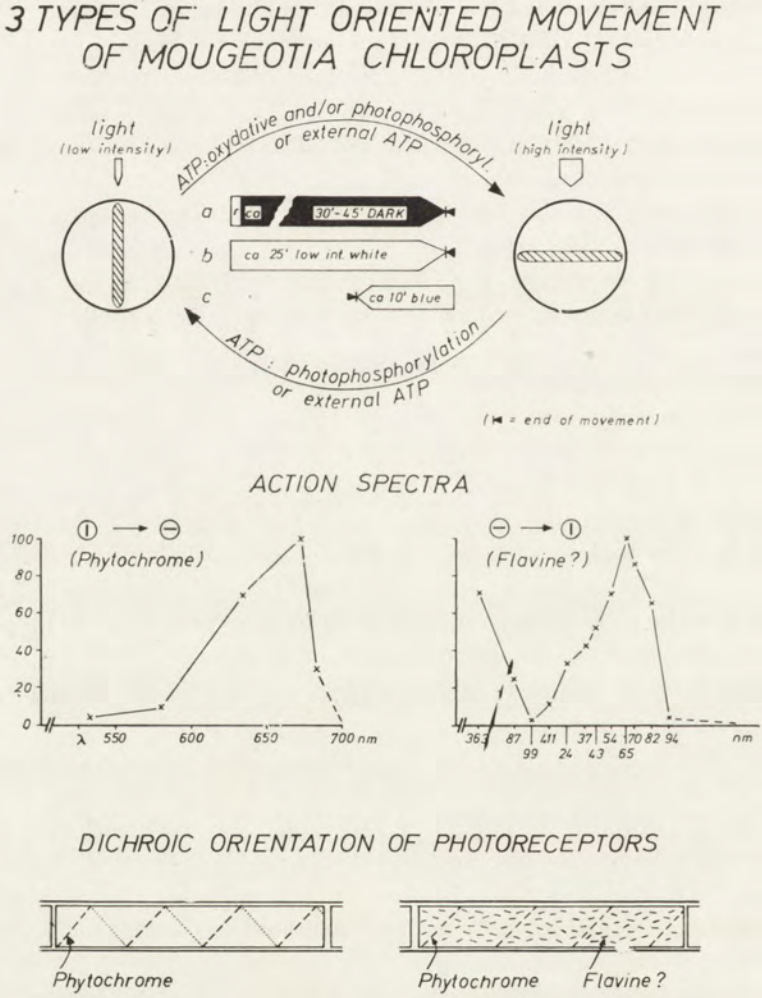
in the plasmic coating not so much of the cell wall as of the chloroplast itself. Some cells of the leaflets of the moss *Funaria hygrometrica* are shown for instance, in Plates I, III and V.

(2) Figure 1 gives a short survey of only few results obtained in former experiments:

(a) The following three types of light-oriented chloroplast movements are known in *Mougeotia*: two types of low intensity movements, that is the movement from profile to plane position (a, b), and one type of high intensity movement, that is the movement from plane to profile position (c). The chloroplast needs 30 to 45 min for the

Fig. 1



turn from profile to plane position in the induction type (a), the movement is induced by a short period of low intensity light – the oriented movement itself taking place in darkness! In type "b" the chloroplasts are moving oriented during a low intensity white light period of about 20 to 25 min. Up to now, the quickest movement revealed in light-oriented chloroplast arrangement takes place in high intensity light, the chloroplast requiring about 6 to 12 min for the rotation from plane to profile in high intensity light (c).

(b) What is known about the energetics of chloroplast movement? Summarizing, we can say that the type "a" movement receives ATP from oxidative phosphorylation only (Fetzer 1963, Schönboh m 1969 a, 1970, 1971 unpublished), type "b" is controlled by oxidative phosphorylation and /or cyclic photo-phosphorylation, whereas type "c", the movement in high intensity light, receives ATP only as may be presumed from the process of cyclic photophosphorylation (Schönbohm 1969 a, 1970, 1971 unpublished). An interesting and important result is, I think, that external given ATP is able to substitute ATP produced by the cell itself (Schönbohm 1969 a, 1970). These results seem to indicate that for realizing different types of oriented chloroplast movement (movement to plane or to profile position!) it is not important where ATP needed for the energetics of movement comes from (compare these results with the hypothesis of Seitz 1971, and in this book page 225).

(c) Let me now come to the action spectra. Action spectra only are known for the induction type "a" of chloroplast movement (Fig. 1, middle – on left hand) and for the high intensity light movement (on right hand). I have to mention that in both types ("a" and "c") phytochrome is a photoreceptor active in perception of the orienting stimulus, whereas for a negative response of the chloroplast (i. e. the high intensity light movement) simultaneously given short wave light of high intensities has to be absorbed in a flavine (Schönbohm 1963, 1966, 1967). Both photoreceptors are localized in the corticalplasm of the cell (Fig. 1, at the bottom), the phytochrome molecules are oriented screwlike (Haupt and Schönbohm 1970), whereas the molecules of flavine (s) are oriented about parallel to the long axis of the cell (Schönbohm 1968, 1971).

(3) Now I have to come to the hypothesis of the mechanism of chloroplast movement in *Mougeotia*: We suppose that contractile fibrillar or thread-like plasmic elements are active in generation of a motive force needed for oriented chloroplast movement; we think that these postulated elements are ATP-ases and sensitive to SH-blockers, as it could be shown for instance in actimyosine.

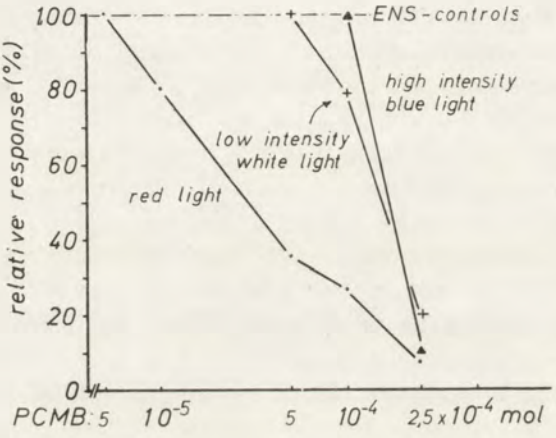
First results of an analysis of plasmic structures (obtained with an interference-phase-contrast microscope) are shown in Pl. II. Here you can see thin and thick fibrillar plasmic elements connecting the plasmic coating of the cell wall with the coating of the chloroplasts, these elements going partly through the vacuole (A–C) and being partly (these filaments) found in the plasmic parietal coating of the cell wall (D, E). We suppose that these fibrils are bundles of contractile actomyosine-like molecules.

(4) Now we will see whether or not chloroplast movement can be inhibited by SH-group blockers.

Figure 2 shows the effect of SH-blocker, PCMB [1] on all 3 types of light-oriented chloroplast movement. The induction type seems to be more sensitive to PCMB (curve: red light) than the two types which need continuous light for movement. The extent of inhibiting chloroplast movement by these differences results from differences in incubation time. As Fig. 3 shows a high inhibitory effect in high intensity light movement is also obtained with PCMB of relatively low concentrations only

Fig.2

Effect of PCMB on light-oriented chloroplast movements



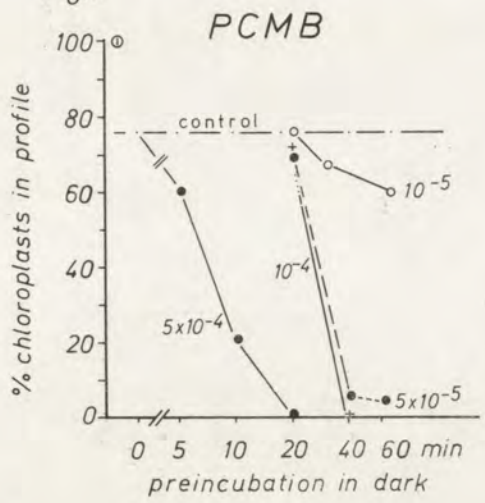
by prolonging incubation time. PCMB has no significant high inhibitory effect if the concentration is reduced to about 10^{-5} mole (M). These results are consistent with those obtained in experiments of the movements of different organisms (Abe 1963, 1964).

Now we have to see, whether PCMB inhibits all 3 types of chloroplast movement via blocking SH-groups. If SH-groups are given for instance by incubating the material with cysteine of a molarity higher than the concentration of PCMB, then the inhibitory effect of PCMB on chloroplast movement is reversed (cf. Table 1 A with B), whereas serine does not have this effect (C). The same results are obtained when PCMB + cysteine are given simultaneously as a mixed solution (E, F).

We have to be cautious in interpreting these results: Izawa and Good (1969) have shown that PCMB inhibits ATPase active in the photosynthetic apparatus

[1] PCMB — p-chloromercuribenzoate.

Fig.3



of chloroplasts but the extent of inhibiting of this ATPase by PCMB increases only to 50–60% and never to 100%. That is an important results. In the PCMB-experiments with *Mougeotia* we received an inhibitory effect up to 100%. ATP does not weaken the effect of PCMB (Table 1 G) so we suppose that PCMB does not inhibit chloroplast movement by effecting the energetic but rather by inhibiting the mechanism. Let us show whether or not ATP has any effect on chloroplast movement of *Mougeotia* preparations being incubated with PCMB of very low non-inhibitory concentrations (Fig. 4, dotted line!). As it is shown in Fig. 4 ATP of non-inhibitory molarities has an inhibitory effect on chloroplast movement if it is given simultaneously with PCMB (10^{-5} m). This effect was obtained for all 3 types of chloroplast movement and could be reversed by cysteine but not by serine (Schönbohm 1969 b). These "PCMB–ATP" results obtained in experiments with *Mougeotia* are in coincidence with experiments of Kriszat (1954) and Portzehl (1954). An exact interpretation of the PCMB–ATP effect should not be tried here.

Let us have a look at the effect of SH-blockers on chloroplast movements of the moss *Funaria hygrometrica*. As can be seen in Pl. III, in each cell of the leaflets of *Funaria hygrometrica* there are a lot of chloroplasts. Darkened for about one hour the chloroplasts have reached dark position (Pl. III). If the material is incubated with a nutrient solution (ENS), then the chloroplasts move to a low intensity light position (B); this movement can be inhibited by PCMB (C). The PCMB effect on chloroplast movement can be weakened by cysteine (E) but not by serine (D).

The micrographs shown in Pl. IV demonstrate that not only relatively thick fibrillar elements can be seen (as shown in Pl. II) but also a fine network of thin fibrils and threads localized in the parietal coating of the cell wall.

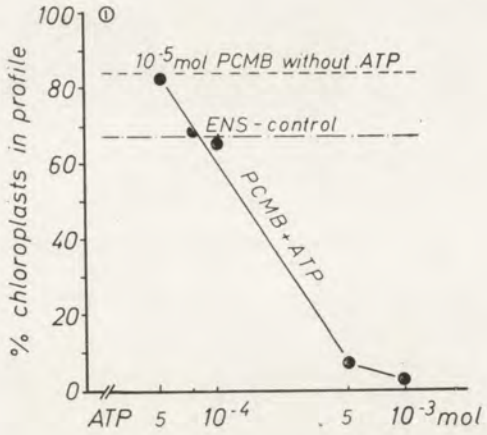
I think that the results shown above do not contradict the hypothesis that contrac-

Table 1

Inhibition of chloroplast arrangement by PCMB and its reversion by cysteine
(High intensity light movement)

Low intensity light (preorientation in plane) 24 min	Sensitizing dark-phase		High intensity light (profile-orientation) 12 min	Response: % chloroplasts in profile	Remarks
	25 min	10 min			
incubation programm					
Phase 1	Phase 2		Phase 3		
A ENS	PCMB (5×10^{-4} m)		PCMB (5×10^{-4} m)	2	inhibition
B ENS	PCMB		cysteine (5×10^{-3} m)	62	inhib. reversed
C ENS	PCMB		serine (5×10^{-3} m)	14	inhibition
D ENS	PCMB		cysteine (10^{-4} m)	16	inhibition
E ENS	ENS		PCMB (5×10^{-4} m)/cysteine (10^{-3} m)	68	inhib. reversed
F ENS	ENS		PCMB (5×10^{-4} m)/cysteine (10^{-5} m)	23	inhibition
G ENS	ENS		PCMB (5×10^{-4} m)/ATP (5×10^{-4} m)	3	inhibition
H ENS	ENS		cysteine (5×10^{-3} m)	60	control 1
I ENS	ENS		serine (5×10^{-3} m)	58	control 2

Fig. 4



tile proteins are active in the mechanism of chloroplast movement and sensitive to SH-blockers, rather we may suppose that these results support our hypothesis.

(5) We will try to answer the following questions:

(a) Will it be possible to influence reversibly the extent of fastening of chloroplasts to cytoplasmic parietal coating by SH-blockers which should be able to inhibit reversibly the contraction of actomyosine-like proteins?

(b) Will it be possible to influence fastening by dark or light periods?

(c) Are the fine plasmic elements, which we have seen in micrographs of Pl. II, IV active in fastening chloroplasts to cytoplasmic parietal coating?

That should be enough for this moment!

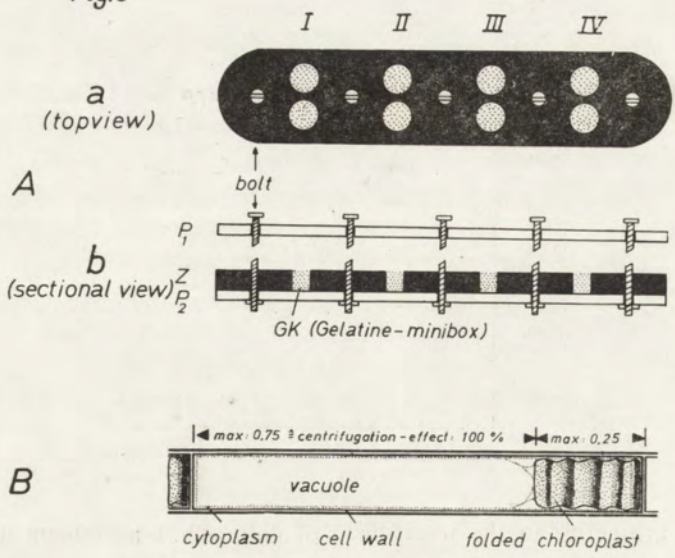
To answer these questions by detailed experiments we undertook centrifugation experiments. The experimental arrangement is shown in Fig. 5. In "A" you can see in top view the middle part with 8 gelatine boxes for fastening the *Mougeotia*-material (a). Clear plexiglass slides are at the top and at the bottom (b). The three parts are held together by 5 bolts. This arrangement is centrifuged for 3 min. The centrifugation forces increase from pair I to pair IV.

In "B" you can see a drawing of a *Mougeotia* cell which has been centrifuged to a high but not damaging extent. The cytoplasmic coating of the cell wall seems to be unchanged, whereas the chloroplast is highly folded. Three quarters (0.75) of the cell are chloroplast-free, this centrifugation effect being defined in test evaluation as 100% centrifugation effect.

(6 a) In the following experiments we tested first the effect of the SH-blocker PCMB on fastening of chloroplast to cytoplasmic parietal coating.

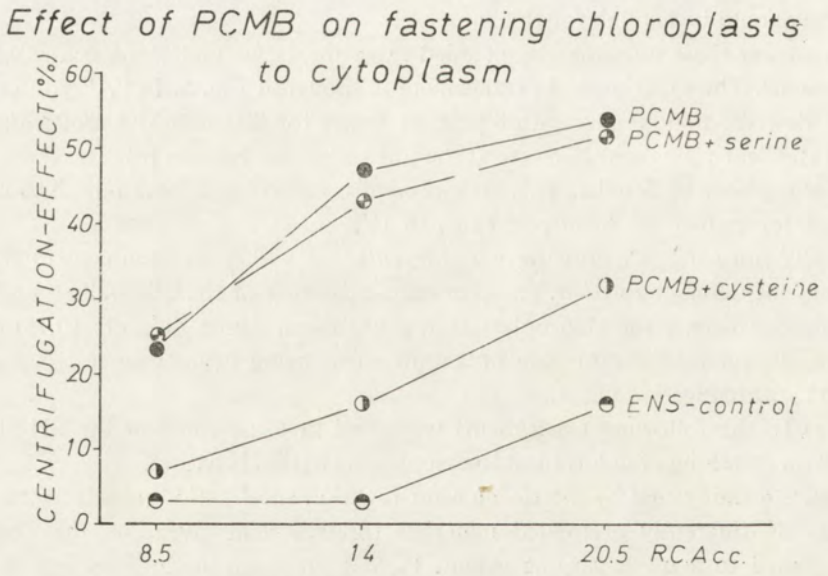
Figure 6 shows that by increasing centrifugation acceleration (abscissa) the chloroplasts of differently pretreated materials (pretreatment given at the curves!) get loosened to a quite varying extent: PCMB–pretreatment loosens considerably

Fig.5



fastening of chloroplasts to cytoplasmic parietal coating, whereas the chloroplasts of the control are fastened to a relatively high extent. The effect of PCMB on the extent of chloroplast fastening in the cell can be weakened dramatically by cysteine but not by serine; we can conclude that this PCMB effect is SH-blocking-effect,

Fig.6



too, just as it could be demonstrated above for the PCMB-effect on light-oriented chloroplast movement.

Quite similar results to those for *Mougeotia* were also obtained with the moss *Funaria* as a test object in centrifugation experiments. In Pl. V you see on the left the position of the chloroplasts (A–D) of material which was incubated after a dark period with different solutions (shown by arrows!) and which was irradiated during incubation period for one hour with low intensity while light. On the right hand you can see the effect of different pretreatments (shown at the arrows) on fastening of chloroplasts to the cytoplasmic parietal coating: only the chloroplasts of the cells incubated with PCMB or PCMB /serine get loosened and centrifuged to the centrifugally oriented part of the cells (B, C), but not the chloroplasts of the control (A) or the PCMB-cysteine-treated material (D). One more result ought to be discussed here: As you can see, the chloroplasts of the control leaflets (A) changed their position during the centrifugation period, namely, from a low intensity light position (A – left side!) to "dark-position" (A – right side) although the dark period in the experiment lasted too short for a common oriented dark movement. We suppose that only light-activated processes are able to fasten the chloroplasts in a low intensity light position. We believe that acceleration forces are able to overcome these processes and can promote the movement in a stable position, which seems to be a dark position of the chloroplasts. This effect of PCMB on changing of the extent of fastening chloroplasts to cytoplasm can also be demonstrated by the present author when the chloroplasts are in dark position (Pl. VI B, C). If we centrifuge such leaflets then the chloroplasts get highly loosened in the PCMB-material (C–E), but not at all in the cells of the controls (B–D).

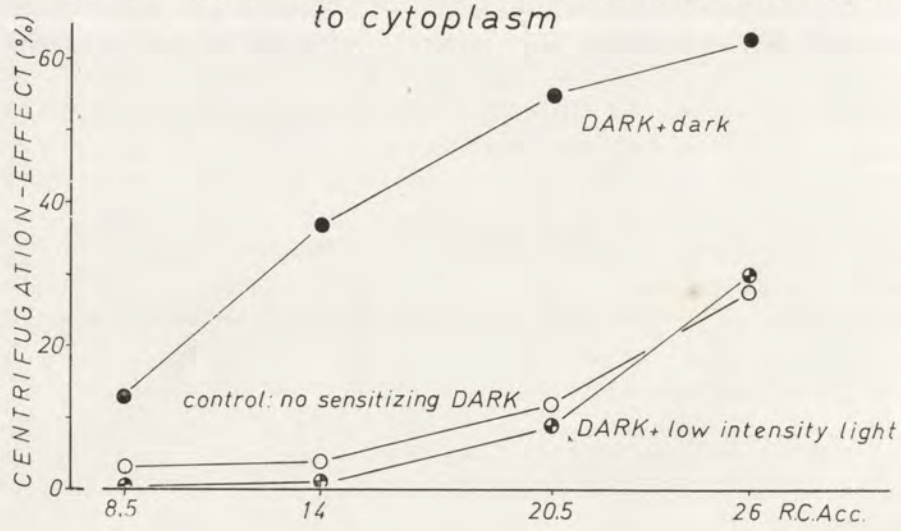
(6 b) Let us come back to *Mougeotia*, our standard object. No specific dark position of the chloroplast of *Mougeotia* is known, but for all 3 types of oriented chloroplast movement a dark-phase has a sensitizing effect on the following light phase. The questions are: What is happening during darkness? What happens to the postulated fibrillar elements, which should be active in the mechanism of chloroplast movement?

In Fig. 7 you can see, that the centrifugation effect is highly increased by a dark period of one hour (upper curve), whereas the chloroplasts of the material which has not been pretreated by a sensitizing dark period are fastened to a high extent in the cell (control). If low intensity white light is applied immediately after the dark period for only a few seconds, then the chloroplasts get highly fastened again to the parietal plasmic coating of the cell within some seconds (see curve at the bottom of Fig. 7).

Quite similar results to those shown in Fig. 7 were also received by experimenting with the material which had been kept in streaming nitrogen or pure oxygen during a dark period of 24 h. In centrifugation experiments we could not find any significant differences in the effect of O_2- or N_2-pretreatment on fastening of chloroplasts. Therefore we do not suppose that a distinct or significant ATP level in the cell is one

Fig.7

Effect of dark and light on fastening chloroplasts to cytoplasm



of the most important facts which have to be realized in the oriented chloroplast movement of a specific type (see also Schönbohm 1969 a).

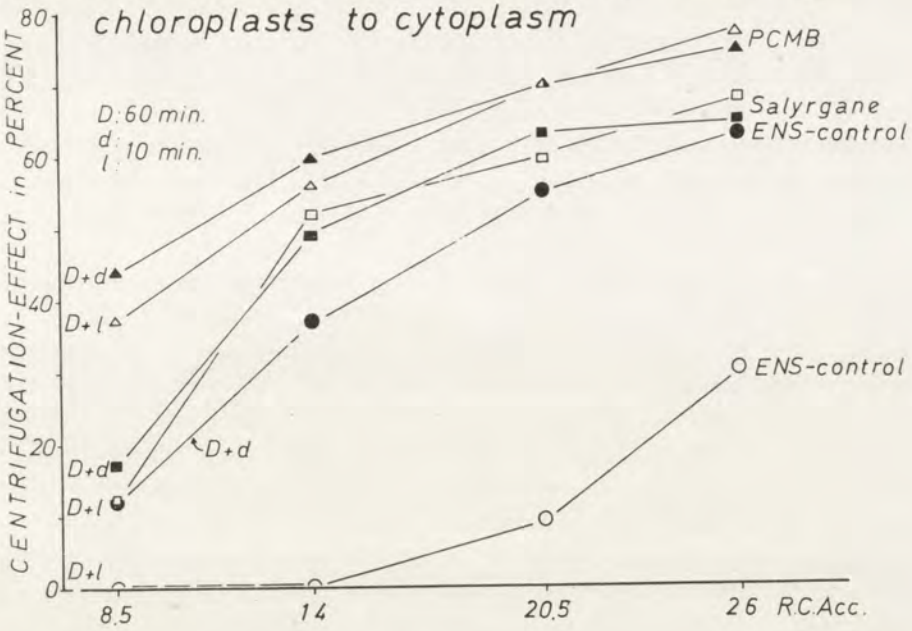
The question which has to follow is whether or not PCMB or another SH-blocker may inhibit light-induced increase of fastening of chloroplasts to cytoplasmic parietal coating – especially in material which has been sensitized by a dark period of about one hour. The answer is given by experiments, the results of which are shown in Fig. 8. You can see three pairs of curves: an ENS-pair (controls), a PCMB-pair and one pair showing the effect of salyrgane [2]. The results obtained with dark preparations of each pair are given in curves with dark symbols, the results of material pretreated by a dark phase and a following low intensity white light phase are shown by curves with open symbols. The curves of the ENS controls demonstrate the expected results. But the two other pairs of curves show that a light period given after the dark phase cannot induce any increase of chloroplast fastening in cells of the material which has been pretreated by incubation with the SH-blockers PCMB or salyrgane. We conclude that SH-blockers are able to inhibit light-induced contraction of fibrillar elements. This results supports our hypothesis, too. Now let us try to look for light-induced establishing of fibrillar or thread-like plasmic elements.

(6 c) If the material is dark-sensitized for at least one hour, then often we do not find any fibrillar elements by the interference-phase-contract microscope technics

[2] Salyrgane: Na-salt of o-{[3(Hydroximercuri)-2-methoxypropyl] carbamoyl} phenoxiacet-acid.

Fig.8

Effect of SH-blockers on light-induced fastening chloroplasts to cytoplasm



(Pl. VII, at the top!). Low intensity white light induces the establishing of fibrillar elements connecting the plasmic coating of the chloroplasts with the parietal coating of the cell wall (Pl. VII, second micrograph). The following micrographs show that the number of the fibrils and threads increases with time and that simultaneously the chloroplast begins to move from profile to plane position (cf. in Pl. VII the micrograph on the top with the micrograph on the bottom).

If dark sensitized material is irradiated with polarized white light then you can see that only few elements get established by light with vibrating E-vector parallel to the cell (Pl. VIII A, D, H). The time from A to C is the same as from A, D to H.

As you can see, polarized low intensity white light with diagonally or perpendicularly vibrating E-vector is not only more effective in inducing light-oriented chloroplast movement but also in the induction of establishing fibrilar plasmic elements than white light with parallel vibrating E-vector.

If you compare the micrographs of Pl. VII with the micrographs of Pl. IX then you will notice, that PCMB-incubation inhibits not only the movement but also to a high extent the increase of fibrilar elements by low intensity white light (Pl. IX A, little stars); if the preperations are irradiated with high intensity light (Pl. IX B, large stars), then only during this light period the number of fibrils seems to increase, simultaneously a quick trembling of plasmic particles and little droplets (only) over a short distance being observed. This PCMB-effect can be partly reversed by

cysteine as it is shown by the micrographs of Pl. X. Here you see some micrographs of one and the same part of a single cell taken once immediately after a dark period (at the top) and then during the low intensity white light period (following pictures). The chloroplast begins to move from profile (top) to plane position (bottom), the number of fibrils increases simultaneously. Pl. XI shows 10 micrographs from a single cell which has been pretreated with PCMB followed by an incubation phase with cysteine. The chloroplast is moving in light; a lot of fibrils and threads can be seen, some fibrils are tortous (H, I, K). (The series A to E demonstrates micrographs taken from the top to the middle of the cell (mediane) and the series E to K shows micrographs taken from the middle of the cell to the bottom part of it).

Now let us consider the Pl. XII of this paper: 4 cells are shown in micrographs taken with a simple phase contrast optic. In "A" the chloroplast (chl) is moving in high intensity light from plane to profile position: you can see a lot of threads and fibrils (f) which seem to pull the chloroplast. In "B" you see with the microscope the cytoplasmic parietal coating of the highest part of a centrifuged cell of its chloroplast-free part (v = vacuole). After the dark period only few fibrils are seen (B). If this cell becomes irradiated with low intensity light (C), a lot of fibrillar elements (f) get established, most of them being oriented perpendicularly and diagonally to the long axis of the cell. Simultaneously we can see parts of an enlarged endoplasmic reticulum (er). In material incubated with PCMB (D) we cannot recognize such elements as could be shown in C. Some elements can be seen oriented parallel to the cell axis; the number of these few elements does not increase during light period.

Our opinion is that the results reported here (and also the results of former experiments: Schönbohm 1969 b) support the formulated hypothesis on the mechanism of chloroplast movement.

We suppose that in *Mougeotia* not only phytochrome but also flavines are able to influence the extent of contraction and relaxation of fibrilar proteins with ATP-ase function.

As we know from experiments performed by Portzehl et al. (1969) contracted ATP-ases are enzymatically much more effective than in relaxed state.

The light-oriented movement is then controlled, so we suppose, by contractile ATPases in different activated states caused by excised photoreceptors (phytochrome/flavines) of the cortical cytoplasm. However, more research is needed before the above formulated hypothesis is fully proved.

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Summary

In a hypothesis it is postulated that contractile fibrillar plasmic elements with ATP-ase function are active in the mechanism of chloroplast movement. This hypothesis is supported by numerous experiments:

(1) All types of oriented chloroplast movement of *Mougeotia* and *Funaria hygrometrica* can be reversibly inhibited by SH-blockers (PCMB).

(2) The extent of fastening of chloroplasts to the plasmic parietal coating of the cell wall can be reversibly decreased by SH-blockers or by a sensitizing dark period. Low intensity white light induces an increase of fastening chloroplasts in the cell.

(3) With an interference-phase-contrast microscope it could be demonstrated, that plasmic fibrils get established by light and that the number of fibrils decreases in darkness. SH-blockers can inhibit the light-induced increase of the number of plasmic fibrils.

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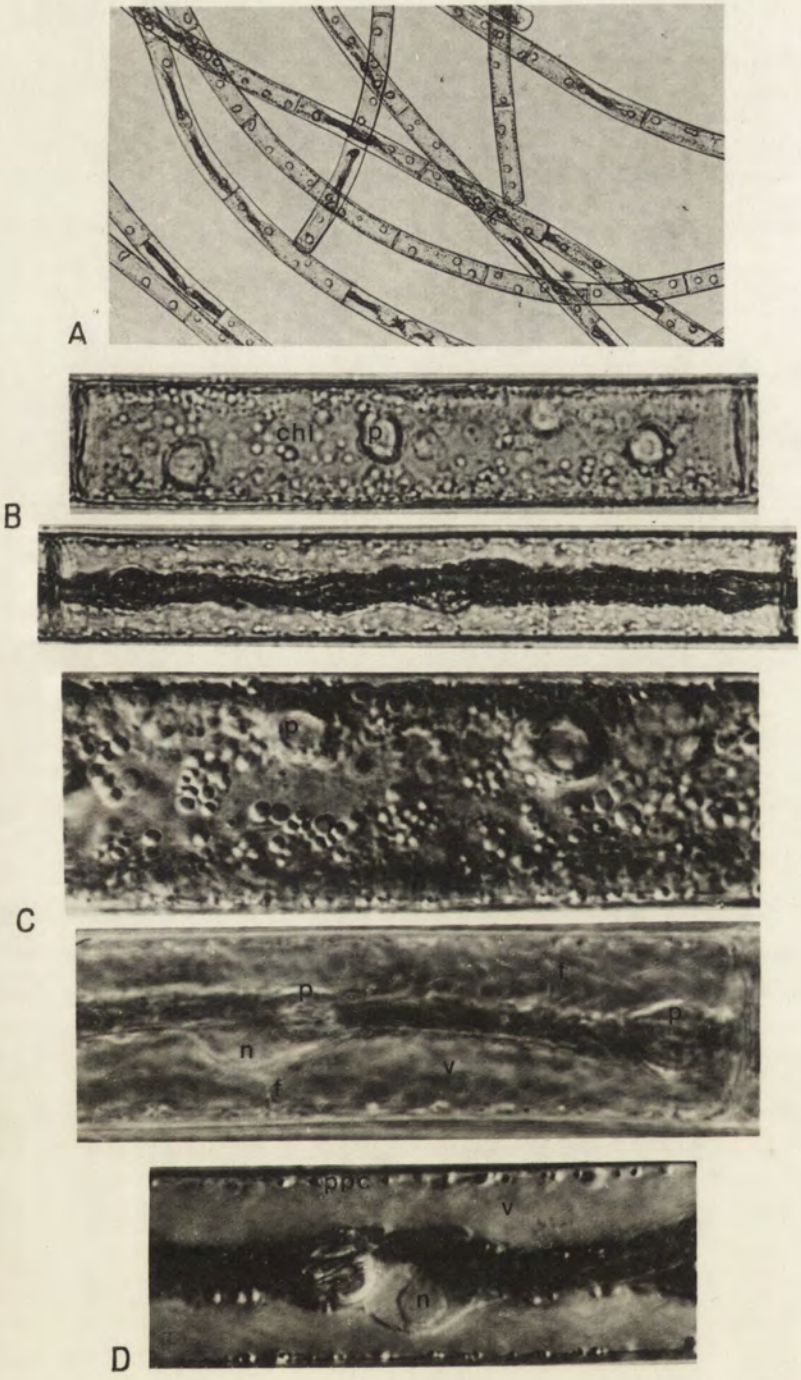
EXPLANATIONS OF PLATES I–XII

All explanations see text



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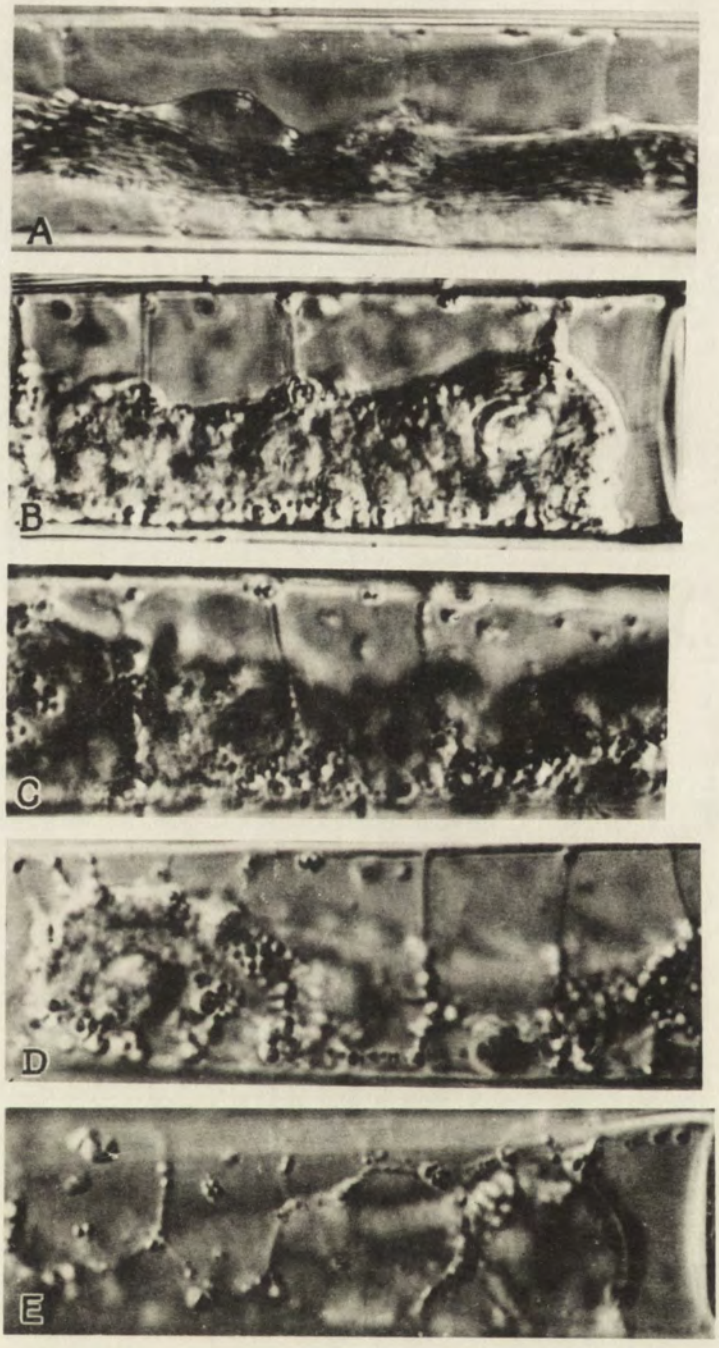

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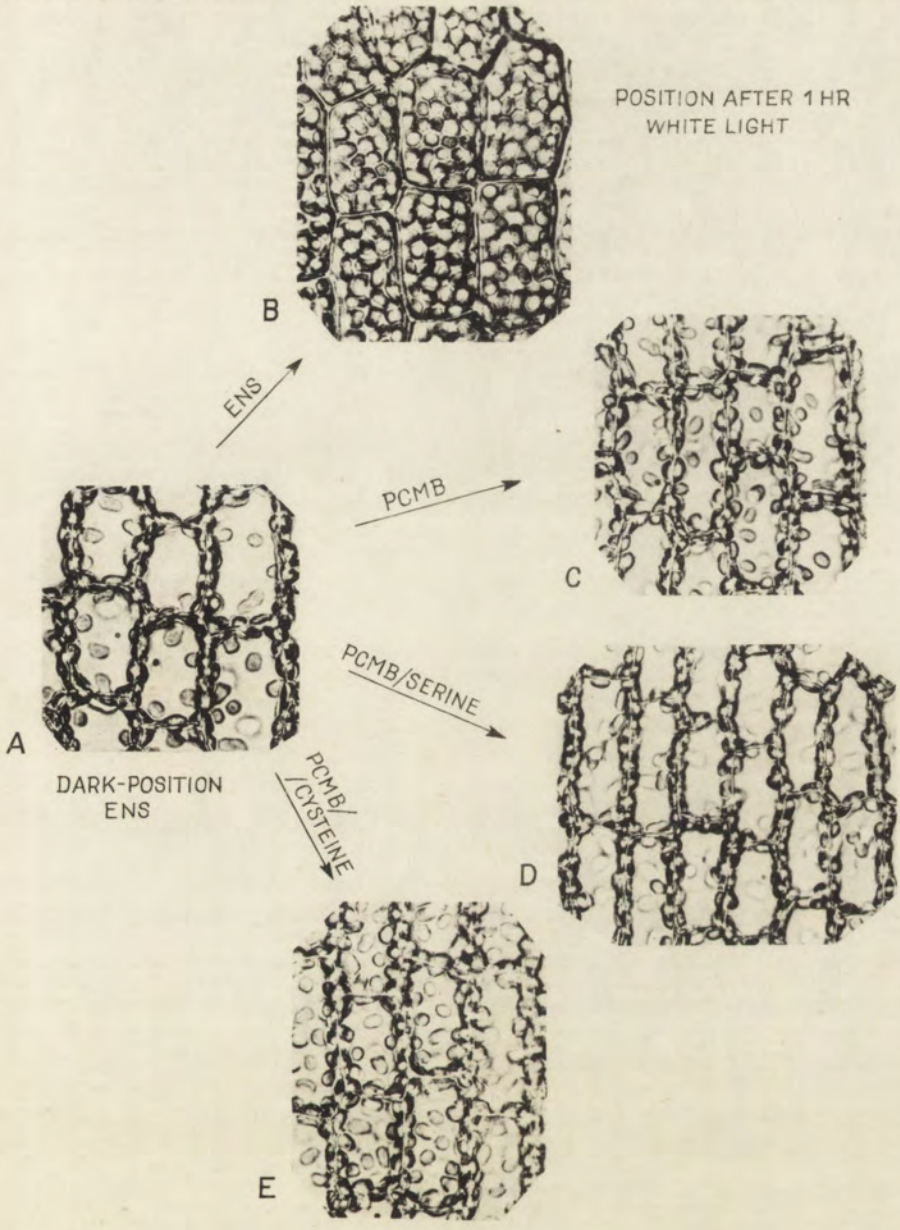

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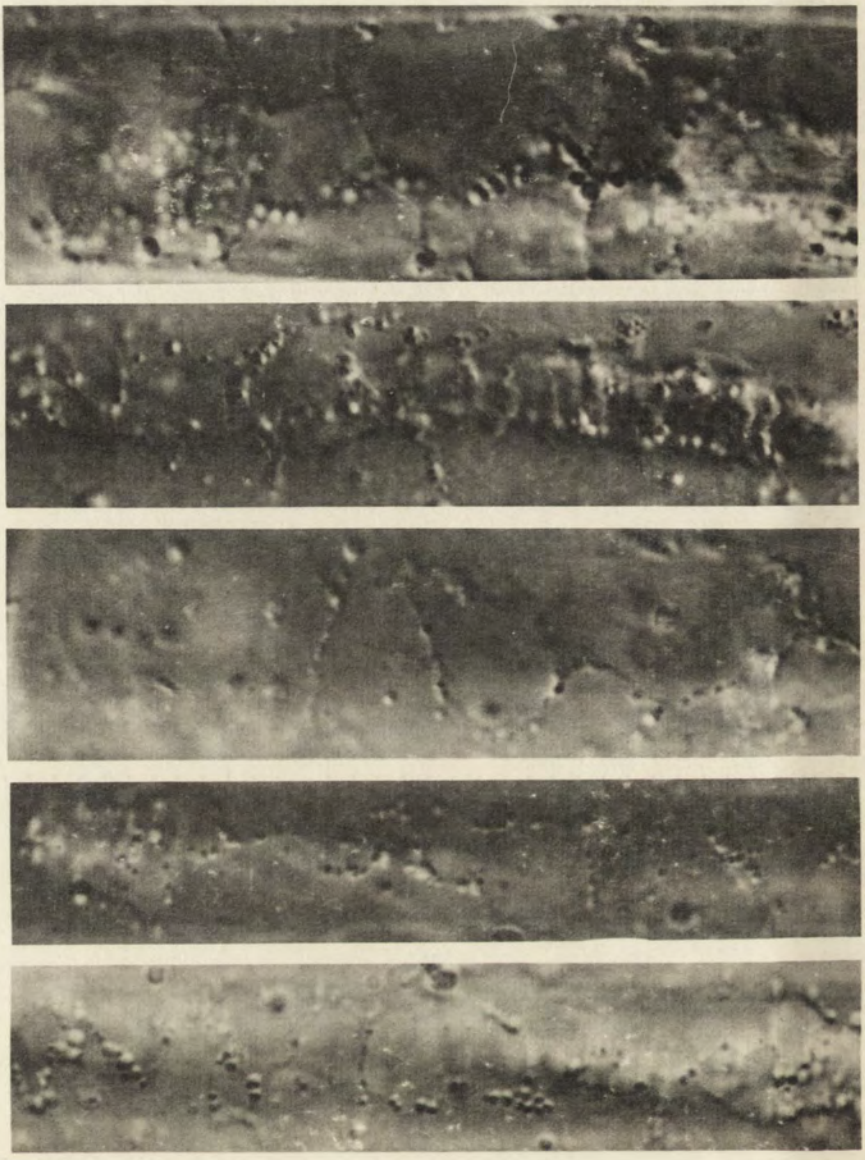

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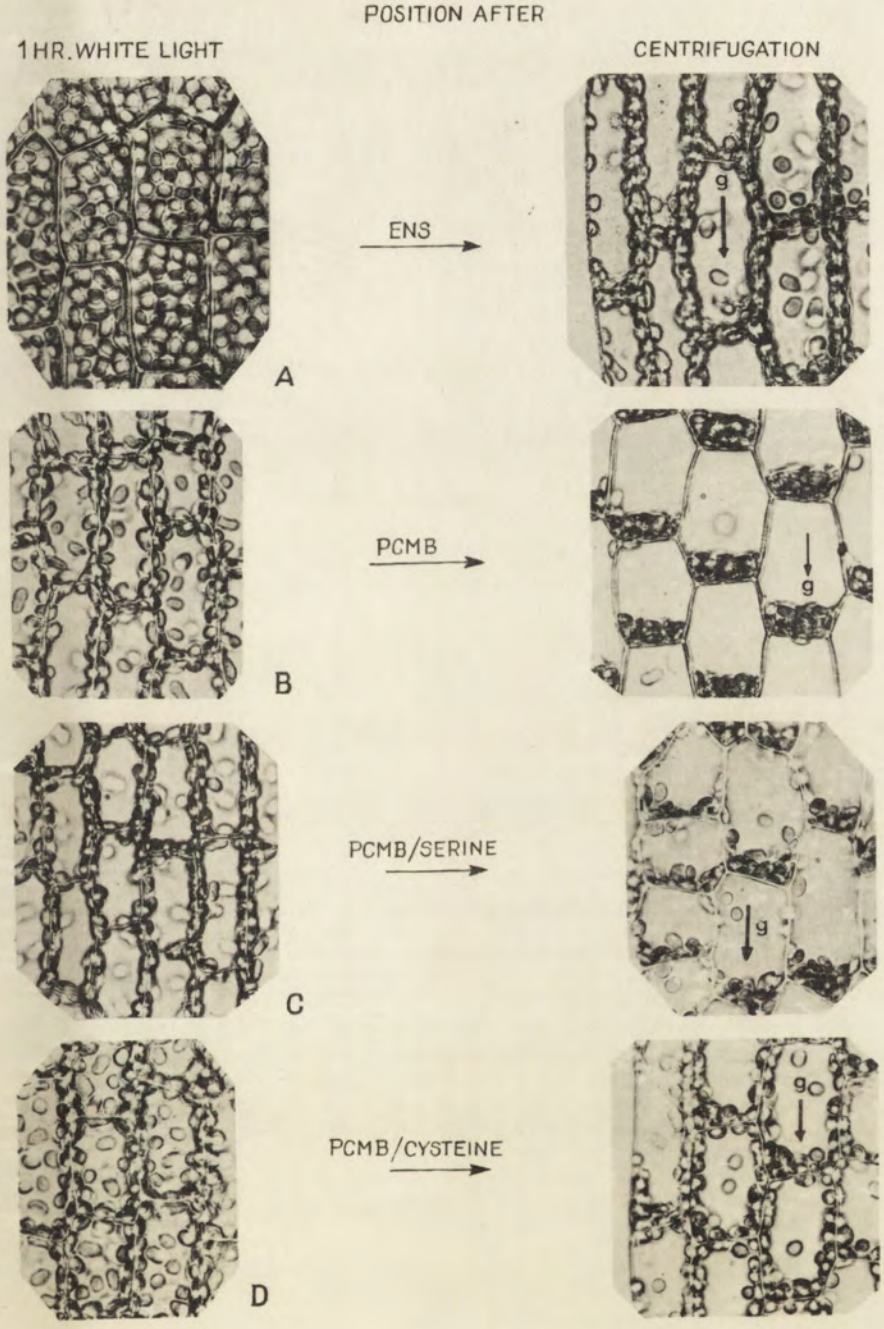

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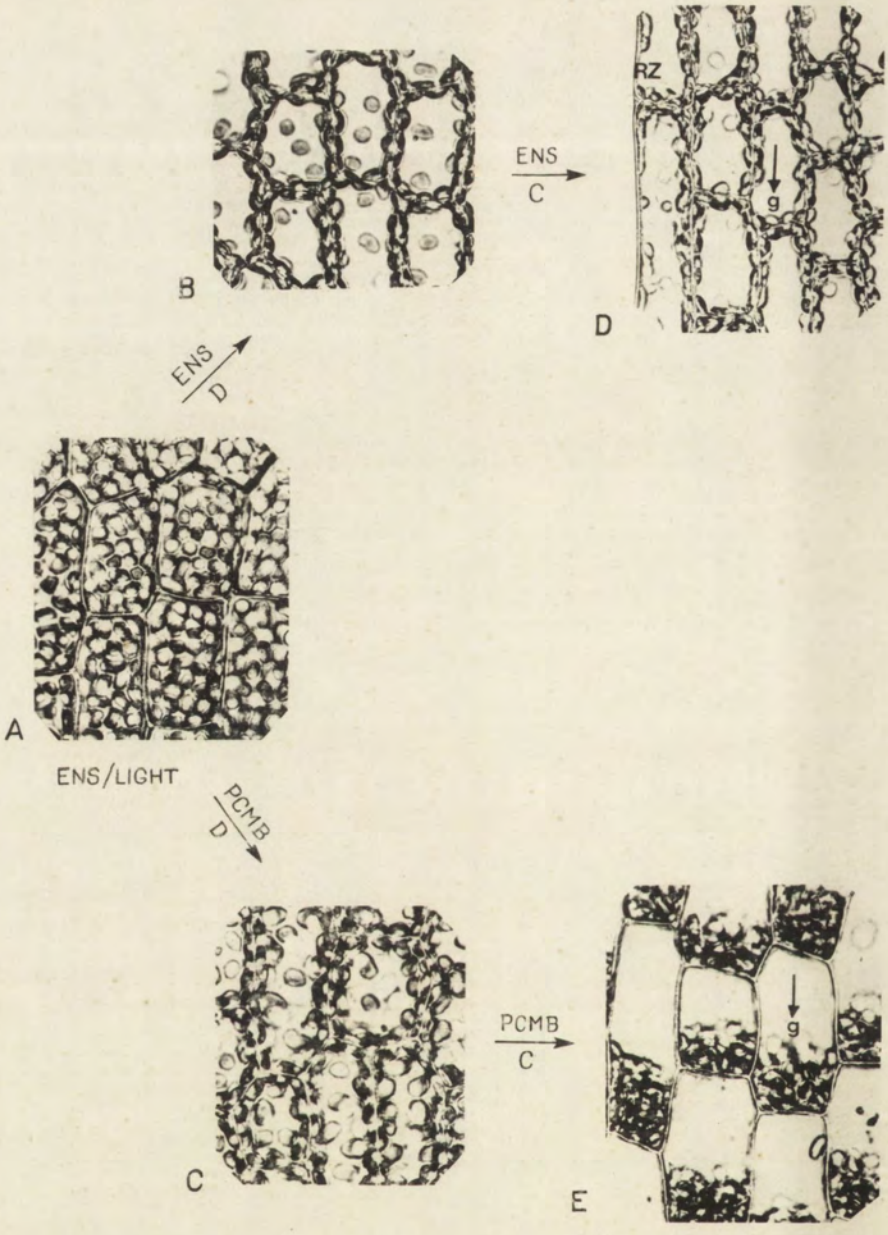

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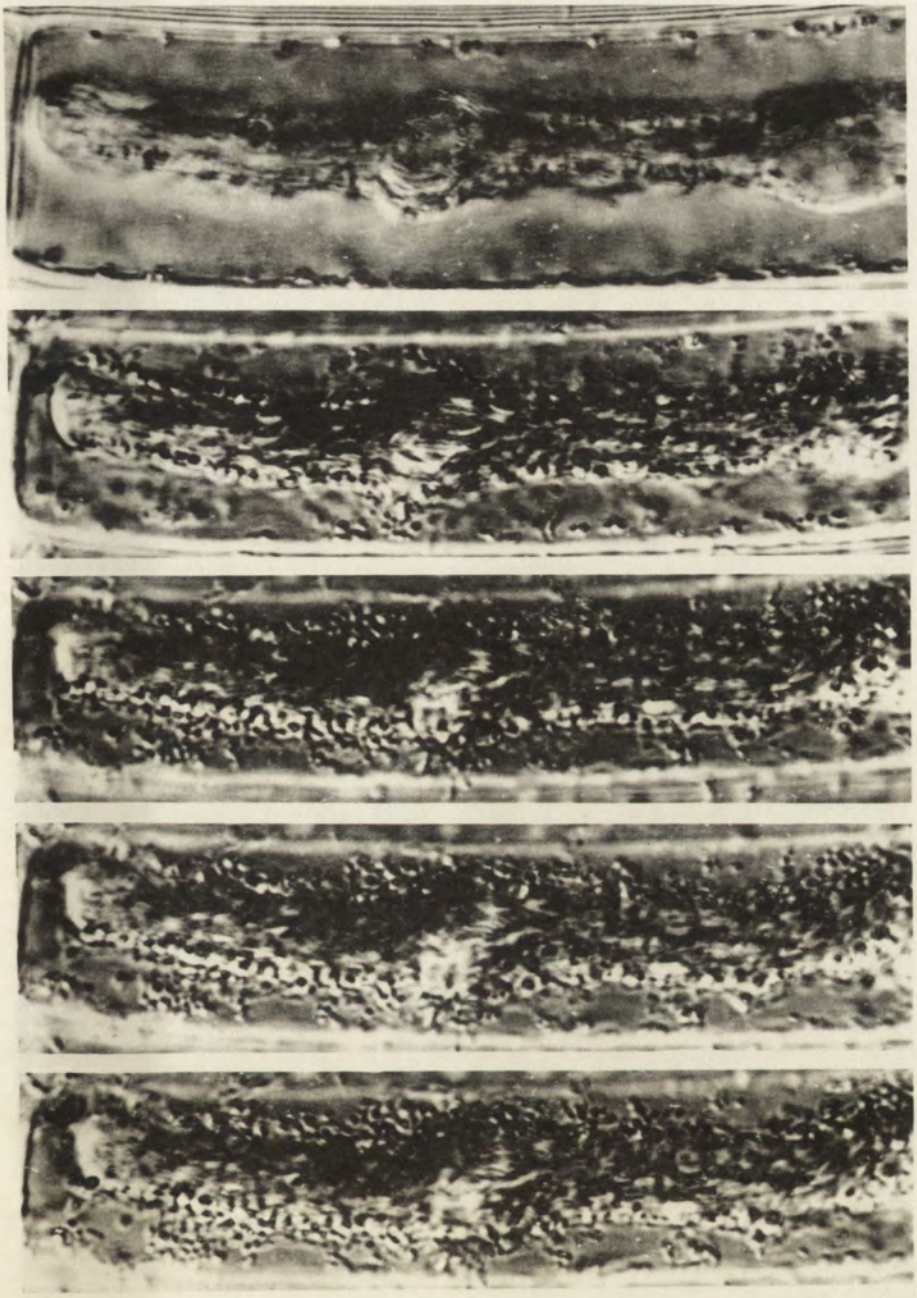

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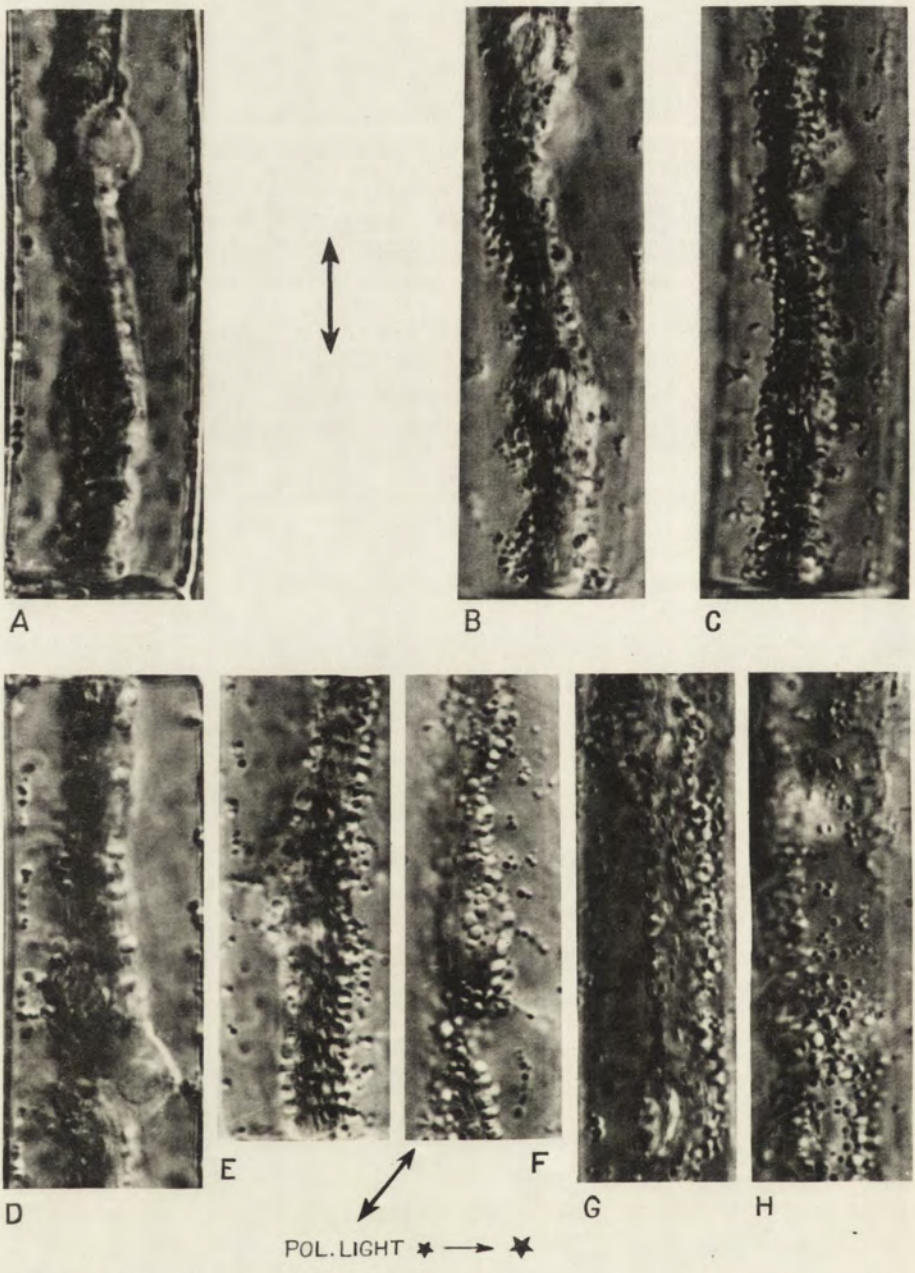

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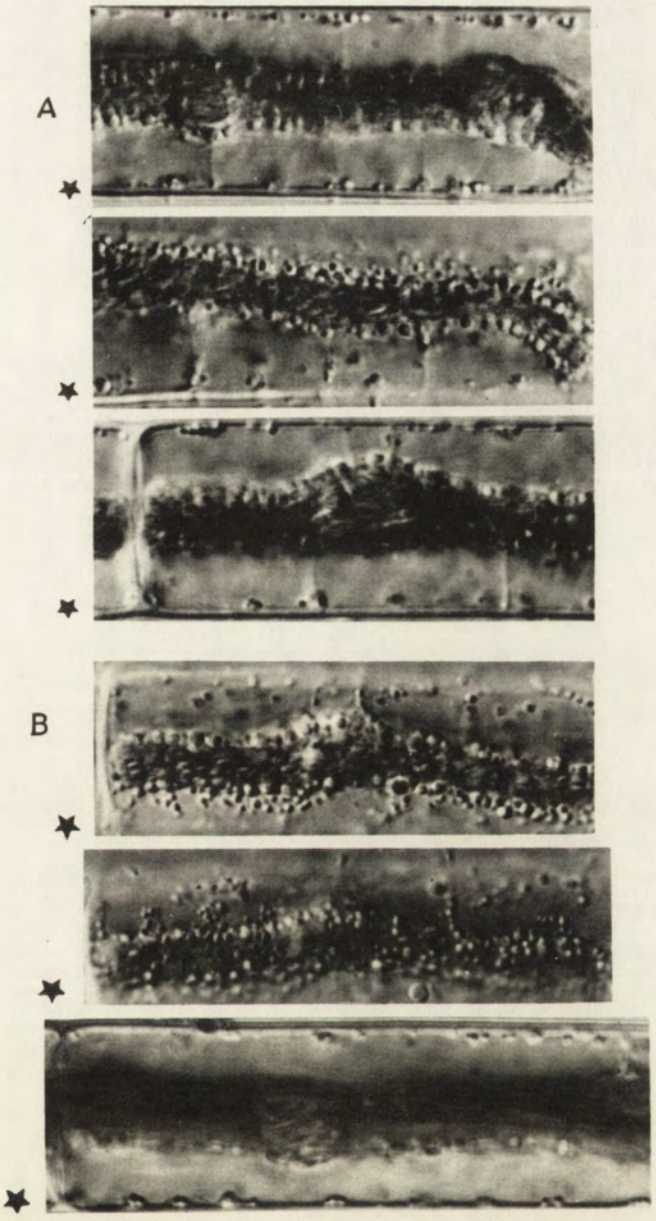

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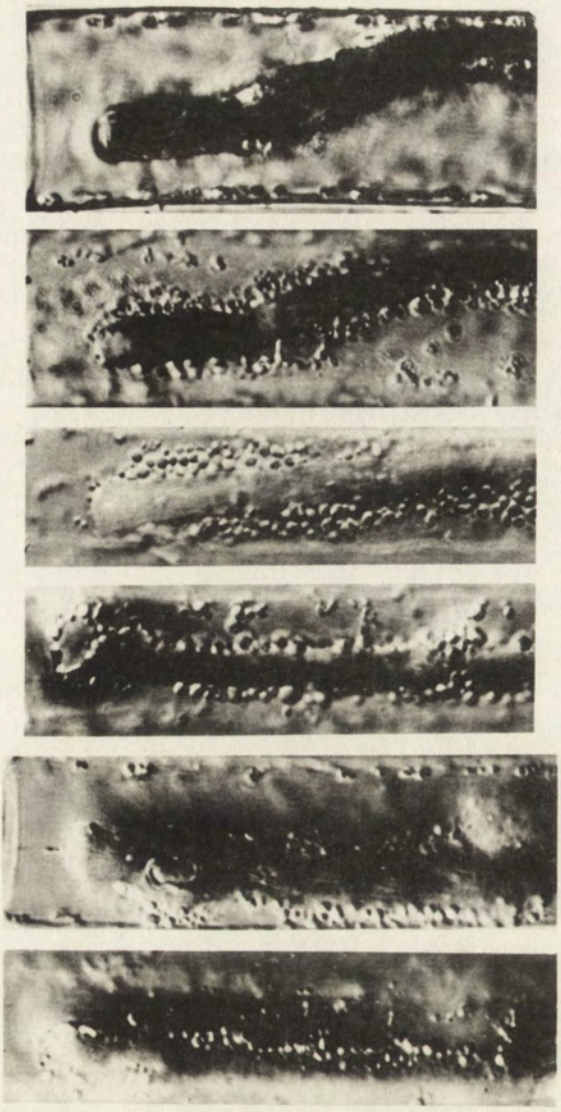

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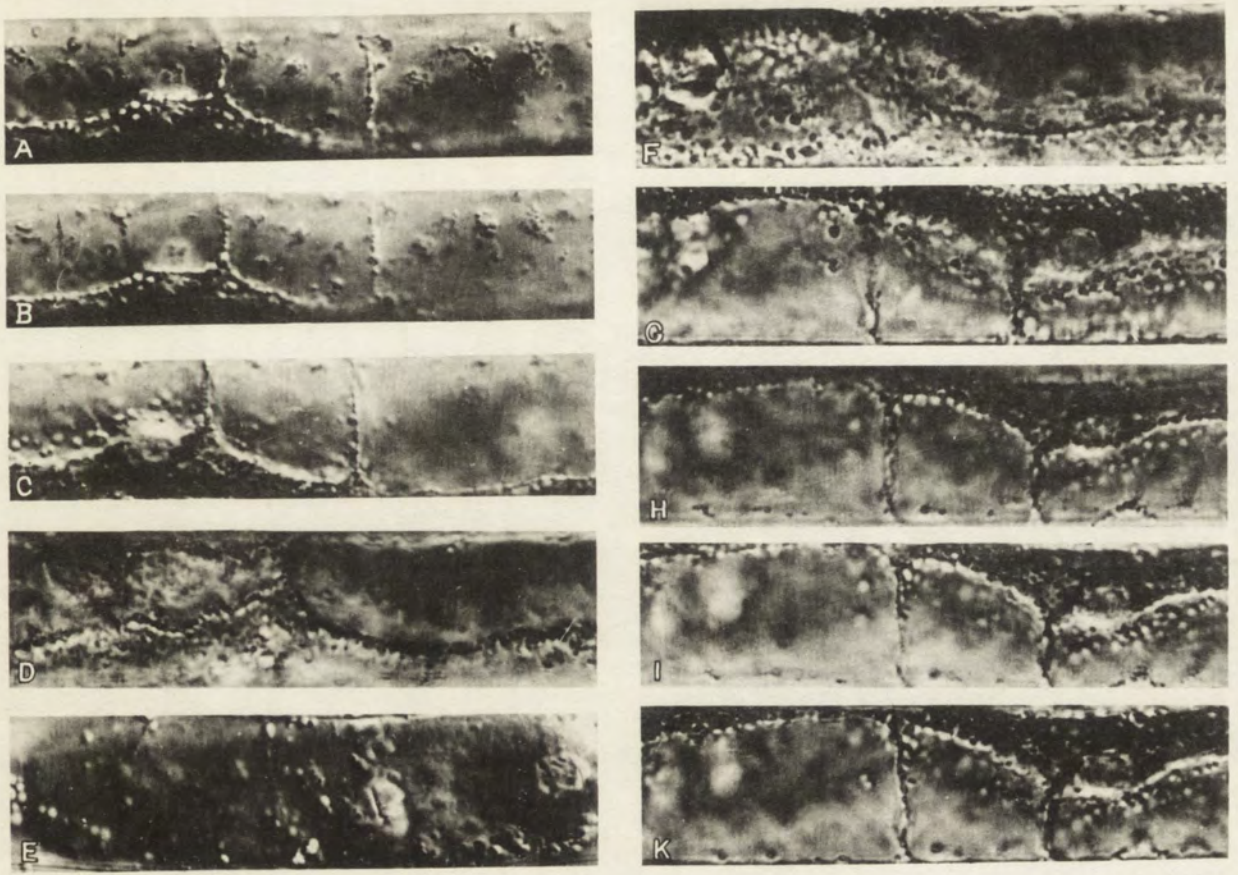

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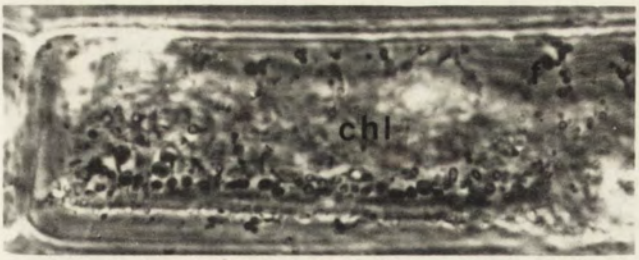

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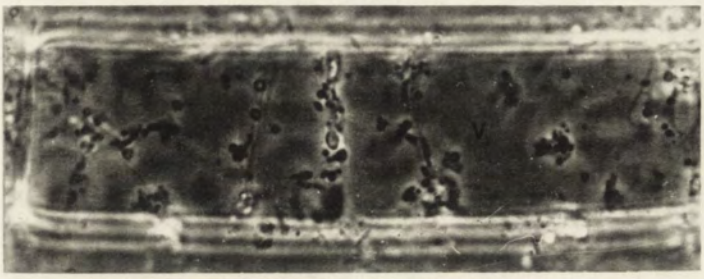

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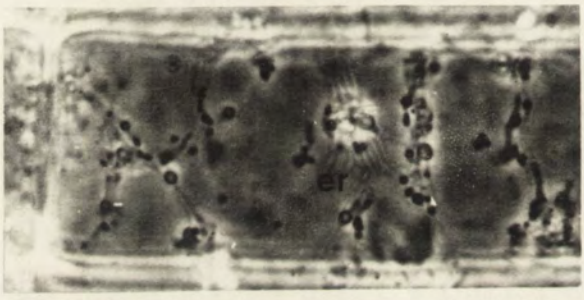

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Primary processes controlling the light induced movement of chloroplasts

In many plants one can distinguish three light dependent movement-reactions of chloroplasts. These are the phototactic orientation movement between the pericline and anticline walls of the cell, the rotation (i.e. cyclosis) in which chloroplasts participate in light accelerated cytoplasmic streaming and the passive light controlled mobility of chloroplasts in the centrifuge.

Phototactic orientation movement of chloroplasts is of special interest, since it possibly represents a regulating mechanism for the control of photosynthesis according to the varying light conditions. Under low or medium intensity irradiation chloroplasts move to the pericline walls, where the incident light can be absorbed optimally. Under high intensity they move to the anticline walls, possibly to find protection against the damaging action of the high intensity, or to take advantage of the light conditions being now optimal for photosynthesis at the anticline walls. In darkness chloroplasts are more or less evenly distributed all over the walls of the cell.

Rotation is induced by high intensity irradiation; here chloroplasts gather at the anticline walls and participate in the rotational streaming of cytoplasm.

In the case of centrifugability, that is passive mobility in a centrifuge, chloroplasts are moved from the pericline and anticline walls to the centrifugal end of the cell. Here three different intensity ranges of the effect of light can be distinguished (Virgin 1951). In comparison to a dark-control light increases centrifugability of chloroplasts at low intensity, decreases it at medium intensity and increases it again at high intensity. According to the literature an increase in centrifugability of chloroplasts corresponds to a decrease in the viscosity of cytoplasm. Yet it is difficult to judge whether the viscosity of cytoplasm as a whole or only that of the contact layer of chloroplasts with the ectoplasm (anchoring bonds) is altered (Zurzycki 1962). So it may be in better agreement with the parameter measured in the experiment (percentage of cells with chloroplasts displaced by centrifugation) to interpret the increase in centrifugability just as an increase in the mobility of chloroplasts. Considering this, centrifugability will be used here as a measure for mobility of chloroplasts in the cell.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

In the following it is intended to analyze the mechanism of the effect of light on the three movement reactions of chloroplasts. In this analysis one is interested in three questions: Which photoreceptor is responsible for the absorption of light? Which processes are initiated? and which causal relations interconnect the three movement-reactions?

The light absorbing pigments

Increased centrifugability, rotation and high intensity movement of chloroplasts in *Vallisneria* are induced by light of the same intensity range. The intensity dependence of the reactions follows a parallel course and saturation is reached at an equivalent level of intensity.

Action spectra of all three reactions have maxima at 370 and 450 nm, which indicates a flavin as photoreceptor. In addition there are smaller peaks at 430, 480, and 680 nm; here light is absorbed in the photosynthetic pigments chlorophyll a and b. The action spectra show a remarkable agreement in the position of peaks and in the amount of incident quanta necessary for the reaction. This indicates that light induces the increase in centrifugability, rotation and high intensity movement of chloroplasts by means of the same primary process (Seitz 1967 a).

Action spectra for the increase in centrifugability and the induction of rotation in *Elodea* have peaks in the same spectral region as well (Seitz 1964, Virgin 1952). In addition the action spectra of high intensity movement in *Lemna* and *Vaucheria* and also the action spectra of low intensity movement in these objects show a good agreement with the action spectra from *Vallisneria* (Haupt 1963).

Summing up one can thus conclude regarding the photoreceptor, that light controls centrifugability, rotation and phototactic movement of chloroplasts by means of the same primary photoreaction. In this primary photoreaction two different pigment systems cooperate. This can be concluded from the position of peaks in the action spectra and from experiments with *Vallisneria* proving a synergism between UV (370 nm) and red; a different effect of inhibitors in UV and red (see Table 1) and a difference between UV and red in the effect of polarized light. In one of these photoreceptor systems a flavin operates in UV and blue, in the other the photosynthetic pigments are active in the blue and red range of the spectrum (Seitz 1967 a, b and 1970).

Interconnection between centrifugability, rotation and phototactic movement of chloroplasts

When analyzing the mechanism of light action one must consider the relation between centrifugability, rotation and phototactic orientation movement of chloroplasts. During their movement in the cell chloroplasts are transported passively by the cytoplasm. This fact indicates that the activity of cytoplasmic streaming is

of great significance for the movement of chloroplasts. The motive mechanism of cytoplasmic streaming is localized at the boundary between endo- and ectoplasm, possibly near the plasmalemma. The motive force is caused by contractile, ATP-consuming filaments, generating a gliding movement of the endoplasm along the ectoplasm (Kamiya 1962). Accordingly, the activity of the motive mechanism should be strictly dependent upon the availability of ATP. The chloroplasts are localized in the endoplasm at the boundary to the ectoplasm. Thus for the movement of chloroplasts the activity of cytoplasmic streaming and especially the activity of the motive mechanism at their surface of contact with the ectoplasm is of significance.

Upon investigation of the effect of light on the rate of cytoplasmic streaming and induction of rotation of chloroplasts the following was found in *Elodea* (Fig. 1). Irradiation increases in dark-adapted cells the rate of cytoplasmic streaming until, depending on the intensity used, after approximately 1–5 min a level of saturation is reached. Chloroplasts start to rotate, that is to move with the cytoplasm in these

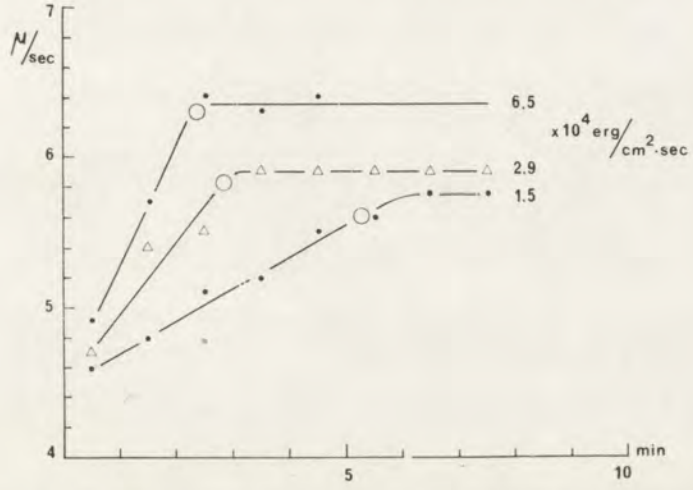

Fig. 1. Increase in rate of cytoplasmic streaming and start of chloroplast rotation (O) in cells of *Elodea*. Values for irradiation of dark adapted cells with different intensities of blue light (BG 28) from Seitz 1964. Ordinate: rate of cytoplasmic streaming, abscissa: time after start of irradiation

cells whenever the cytoplasmic streaming has reached its maximal rate and when, accordingly, the motive mechanism has its maximal activity. Apparently the induction of rotation of chloroplasts is a consequence of an activation of cytoplasmic streaming. This allows to conclude further that also in the case of high intensity movement the beginning of the movement of chloroplasts is preceded by an increase in the rate of cytoplasmic streaming. Chloroplasts are then transported by the cytoplasm into the high intensity arrangement.

Concerning the relation between centrifugability and phototactic movement it is known that centrifugability is increased only during the phototactic movement of chloroplasts (Fig. 3; Virgin 1951, Zurzycki 1964). This indicates that centrifugability is increased only when chloroplasts are already moving with the cytoplasmic streaming. Centrifugability of chloroplasts is therefore like rotation, just a manifestation of the activity of cytoplasmic streaming.

To sum up the three light induced movement reactions of chloroplasts are apparently dependent upon the activity of cytoplasmic streaming as one common primary cause. This agrees very well with the conclusion drawn from the action spectra, which indicated that all three reactions are induced by light through the same primary photoreaction.

The gradient for the phototactic movement of chloroplasts

After the initiation of the movement a gradient is necessary in the cell to orient the course of phototactic movement of chloroplasts. Due to the geometric and optical properties of the cell the incident intensity of light at the anticline walls is very much reduced in comparison with the pericline walls. This difference in intensity produces a gradient of light absorption between the anticline and pericline wall (Zurzycki

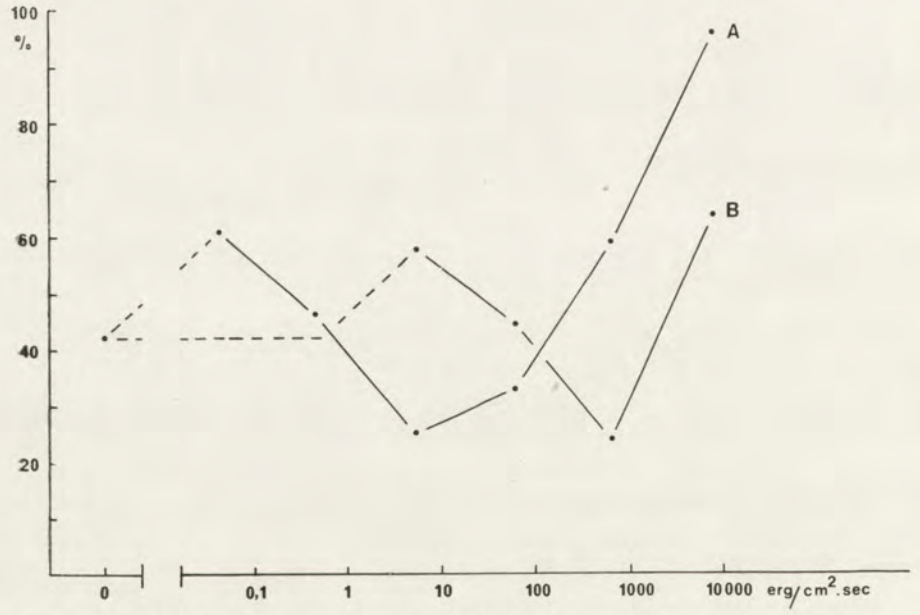

Fig. 2. Intensity dependence of centrifugability of chloroplasts at the pericline (A) and anticline (B) wall of cells of *Vallisneria*. Dark pretreated leaves were irradiated for 20 min with 454 nm. Intensity of this irradiation is on the abscissa, percent centrifugation on the ordinate. Data are slightly schematized from Seitz 1971

1962). Since light alters centrifugability of chloroplasts either by increasing or decreasing it depending on the intensity used, a gradient of centrifugability of chloroplasts can result between these walls.

Experimental results of a separate evaluation of centrifugability of chloroplasts at the anticline and pericline walls in *Vallisneria* are shown in Fig. 2.

The ranges of light induced increase, decrease and again increase in centrifugability of chloroplasts in comparison with a dark control are reached at the pericline and anticline walls of the cell at different intensities of irradiation. The curves indicate a ratio of effective intensity of approximately 1 : 30 between the two walls.

The resulting difference in absorption causes the effect that at high intensities centrifugability of chloroplasts is increased at the pericline wall and at the same time decreased at the anticline wall – a situation which brings about the high intensity movement of chloroplasts. At medium intensities, on the other hand, centrifugability of chloroplasts is decreased at the pericline wall and at the same time increased at the anticline wall – a circumstance which causes low intensity movement of chloroplasts.

Metabolic processes involved

An important point which remains to be analyzed is the question regarding the metabolic processes responsible for the effect of light on the movement of chloroplasts. The after-effect of a short irradiation of high intensity demonstrates here that the increase in centrifugability of chloroplasts is fully established only several minutes after the irradiation (Fig. 3). This indicates that light induces processes which can proceed in darkness and which finally result in increased centrifugability

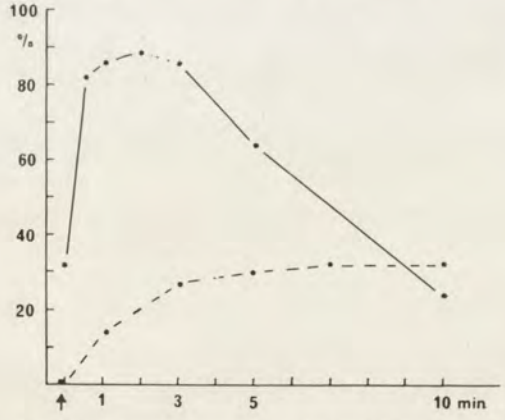

Fig. 3. The after-effect of a short irradiation on centrifugability (——) and high intensity movement (– – – –) of chloroplasts in *Vallisneria*. Irradiation (arrow): 10 sec blue (BG 28), 5×10^6 erg/cm^2 sec abscissa: duration of dark-phase between irradiation and measurement of the reaction, ordinate: per cent centrifugability and per cent high intensity arrangement of chloroplasts. Data from Seitz 1971

of chloroplasts. A reasonable assumption regarding these dark processes seems to be that they are identical with the light induced increase in rate of cytoplasmic streaming, which precedes the movement of chloroplasts (cf. Fig. 1).

Further information about the metabolic processes involved in the mechanism of light action can be obtained from studies with inhibitors.

Table 1

Effect of inhibitors on high intensity movement of chloroplasts in *Vallisneria*

Reaction in per cent of control			
in presence of:		after 20 min irradiation with:	
		UV 369 nm 3.4×10^3 erg/cm^2 × sec	Red 683 nm 5.9×10^4 erg/cm^2 × sec
PCMB (p-chloromercuribenzoate)	10^{-4} M	39	38
DCMU (*3*-(*3,4*-dichlorophenyl) *1*-, *1*-dimethylurea)	10^{-7} M	100	41
DNP (*2, 4*-dinitrophenol)	3×10^{-4} M	37	237
Azide (NaN_3)	3×10^{-3} M	33	130

(Data from Seitz 1967 b and 1970).

Table 1 shows data for the effect of PCMB, DCMU, DNP and azide on high intensity movement of chloroplasts in *Vallisneria*. The movement was induced by irradiation with either UV or red, which allows to make a distinction between the two photoreaction systems involved. The inhibition by PCMB, which is effective in ultraviolet as well as in red light, indicates that for the effect of both photoreaction systems unrestricted sulfhydryl groups are necessary. The inhibition by DCMU, which is effective only in red and not in ultraviolet light, indicates that photosynthetic processes are responsible for the effect of red light. The inhibition by uncouplers in ultraviolet light proves that ATP from oxidative phosphorylation is essential for the movement induced by the flavin-photoreaction system. No inhibition can be observed in red light with the same concentration of uncouplers of oxidative phosphorylation. The fact that the presence of uncouplers stimulates the effect of red light is most probably due to an increased rate of photophosphorylation under these conditions (Seitz 1970). The experiments with inhibitors indicate that the effect of light is strongly dependent upon an unimpaired availability of ATP (see also Schönbohm 1969 and Zurzycki 1965). Thus it may be possible that the primary effect of light is an increase in the availability of ATP. If this were true, it should be possible to replace an irradiation by the addition of ATP from outside.

As can be seen in Fig. 4 it is indeed possible to induce in *Vallisneria* increased

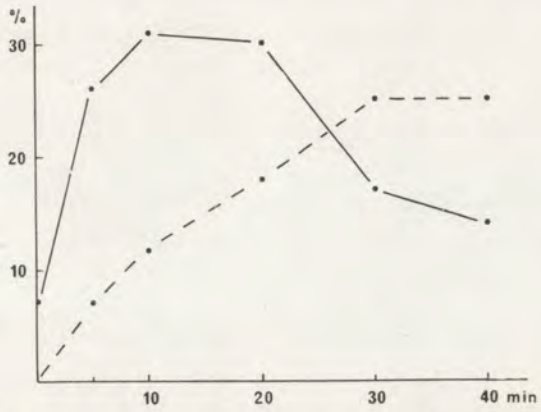

Fig. 4. Induction of increased centrifugability (———) and high intensity movement (– – – –) of chloroplasts by ATP in *Vallisneria*, abscissa: time after adding ATP (10^{-3} M), ordinate: per cent centrifugability and per cent high intensity arrangement of chloroplasts. Data from Seitz 1971

centrifugability and "high intensity movement" by the addition of ATP to the medium.

ATP induces here most probably a stimulated dark movement by neutralizing the low intensity gradient established during the experimental pretreatment (orientation into low intensity arrangement as a starting position for experiments). The time course of the reaction resembles that of the after-effect of a short irradiation of high intensity in so far as centrifugability of chloroplasts is increased only during the orientation movement (cf. Fig. 3).

From the experiments with inhibitors and from the effect of ATP added to the medium it is possible to conclude that the primary process induced by light may be an increased availability of ATP.

The increased availability of ATP would be under control of the two photoreaction systems. In red and blue light of high intensity ATP would be supplied by photophosphorylation. In UV and blue the inhibition by uncouplers suggests the participation of oxidative phosphorylation. Here an irradiation could accordingly stimulate oxidative phosphorylation. An experimental support for this assumption comes from *Chlorella*, where light increases the uptake of oxygen, i.e. the rate of respiration. This effect has, like the light-induced movement of chloroplasts an action spectrum suggesting a flavin as photoreceptor (Kowallik 1969, Ried 1969).

If one compares the intensity dependence of the processes, one finds that the increase in uptake of oxygen, that is the stimulation of oxidative phosphorylation, is induced by UV and blue already at very low intensities and is saturated at medium intensities (Kowallik 1969).

Photophosphorylation on the other hand, which operates in blue and red, begins

at medium intensities. Here one can distinguish two different reactions. These are noncyclic photophosphorylation, which is coupled to electron transport and CO_2 fixation, and cyclic photophosphorylation, which can operate independently from CO_2 fixation. The alternate activity of these two processes is controlled by the availability of NADP (Arnon 1969 a and b). With increasing intensity of light electron transport and noncyclic photophosphorylation are activated first, since electrons go preferentially to NADP and thus into CO_2 fixation. During CO_2 fixation more ATP is consumed than is synthesized by noncyclic photophosphorylation, thus the chloroplasts are forced to take up ATP from the surrounding cytoplasm. This leads to a local shortage of ATP, which would cause a reduced activity of the motive mechanism of cytoplasmic streaming and decreased centrifugability of chloroplasts. Upon a further increase in the intensity of light there comes the range where CO_2 fixation is saturated, turnover of NADP is also saturated and there is no further NADP to take up the electrons. The incident light can now be fully effective in cyclic photophosphorylation. At high intensity of light accordingly chloroplasts synthesize a surplus of ATP, which can pass over into the surrounding cytoplasm, where it can activate cytoplasmic streaming and the movement of chloroplasts. In conclusion one can say that depending upon the intensity and wavelength of light oxidative and photosynthetic phosphorylation together can regulate the availability of ATP at the contact layer between chloroplasts and the ectoplasm.

When comparing the intensity dependence of the effect of light on oxidative and photosynthetic phosphorylation with that on centrifugability of chloroplasts one finds the following.

Low intensity increases the supply of ATP from oxidative phosphorylation and increases centrifugability of chloroplasts. Medium intensity causes, due to photosynthetic CO_2 fixation, a shortage of ATP and decreases centrifugability of chloroplasts. High intensity again increases the availability of ATP from cyclic and oxidative phosphorylation and causes an increased centrifugability of chloroplasts. If one considers further the different incident intensity at the pericline and anticline walls of the cell, that is the gradient of light-absorption, then the experimental facts and the correlations described allow a unifying hypothesis for the mechanism of light-depending movement reactions of chloroplasts.

Availability of ATP as a primary cause of light action

According to this hypothesis a light-induced increase in the availability of ATP would activate the motive mechanism of cytoplasmic streaming and thus induce the movement of chloroplasts. The direction of the movement would be oriented by a gradient of the-availability of ATP, which is built up in the light by both oxidative and photosynthetic phosphorylation in accordance to the difference in the intensity dependence of the effect of light on these processes.

The orientation movement of chloroplasts which possibly might be a mechanism

for the regulation of photosynthesis, as was mentioned in the introduction, would thus itself be controlled by photosynthetic processes in cooperation with oxidative phosphorylation.

The hypothesis will be described in the following for different ranges of increasing intensity of light, actual values given refer to *Vallisneria* at 454 nm (cf. Fig. 2).

In darkness oxidative phosphorylation operates at all walls of the cell equally at a normal dark rate. Cytoplasmic streaming also has its low and constant dark rate. Centrifugability of chloroplasts shows medium values and chloroplasts are spread over all walls more or less evenly in their dark position.

Light of low intensity (ca. 0.1 erg/cm^2 × sec) activates oxidative phosphorylation at the pericline wall only and increases here the availability of ATP, leading to increased rate of cytoplasmic streaming and increased centrifugability of chloroplasts. At the anticline walls there are still dark conditions at this intensity of light. In the resulting gradient of ATP supply a phototactic orientation movement of chloroplasts would be expected. This movement has not been investigated yet.

Light of medium intensity (ca. 10 erg/cm^2 × sec) then activates oxidative phosphorylation also at the anticline walls and leads here to an increased supply of ATP and increased centrifugability of chloroplasts. At the pericline wall the effect of light on oxidative phosphorylation comes into the range of saturation, here photosynthesis begins to operate, first with noncyclic electron transport, CO_2 fixation and ATP consumption. This results in a shortage of ATP and decreased centrifugability of chloroplasts at the pericline wall. In the resulting gradient of availability of ATP low intensity movement of chloroplasts proceeds.

In high intensity (ca. 1000 erg/cm^2 × sec) the effect of light on oxidative phosphorylation is saturated at all walls of the cell. At the pericline wall CO_2 fixation and thus ATP consumption comes into the range of saturation, whilst cyclic photophosphorylation increases further and supplies, together with oxidative phosphorylation, ATP, which activates cytoplasmic streaming and thus increases centrifugability of chloroplasts. At the anticline walls there are now the conditions of medium intensity with ATP shortage and decreased centrifugability of chloroplasts. In the resulting gradient of availability of ATP high intensity movement of chloroplasts is observed.

In very high intensity (ca. 20000 erg/cm^2 × sec) CO_2 fixation is in saturation at the anticline wall, too. So there is a surplus of ATP for the motive force of cytoplasmic streaming and chloroplasts start to rotate as soon as they have reached full high intensity arrangement (Seitz 1967 a).

Discussion

The assumption of an effect of light on oxidative phosphorylation, which is presented here, implies that the flavin photoreceptor is most probably localized in the mitochondria. Thus it appears to be unlikely that this photoreceptor could

be oriented preferentially in respect to the cell walls. This is in contradiction to conclusions drawn from experiments with polarized light, which indicated that the photoreceptor is localized in the ectoplasmic layer and is oriented parallel to the walls of the cell (Haupt 1968, Zurzycki 1967).

An alternative assumption for the intimate connection between the movement of chloroplasts and oxidative phosphorylation, which would reconcile this contradiction, is the following. Light being absorbed in the flavin photoreceptor, which is localized in an oriented way in the ectoplasm, could activate the contractile filaments of the motive mechanism of cytoplasmic streaming, which are localized either in or at the ectoplasmic layer. This activation would cause increased consumption of ATP due to the ATPase activity of the contractile proteins. Uncouplers of oxidative phosphorylation could accordingly inhibit the light-induced reaction by blocking the availability of ATP.

If this alternative mechanism of light action is applicable, it is necessary to assume further that the addition of ATP from outside (Fig. 4) can substitute only the effect of light absorbed in the photosynthetic reaction system and does not replace an effect of the flavin photoreceptor.

Further investigations concerning this problem are in progress. For a more detailed discussion and reference to the literature see Seitz 1971. The work was supported by Deutsche Forschungsgemeinschaft.

Summary

The light induced movement reactions of chloroplasts are analyzed with special reference to results from *Vallisneria*. From the interconnection of the effect of light on centrifugability, cyclosis and phototactic orientation movement of chloroplasts and from experiments with inhibitors of phosphorylation processes a hypothesis regarding the primary process of light action was derived. According to this hypothesis the primary effect of light is a regulation of the availability of ATP from oxidative and photosynthetic phosphorylation. An increased availability of ATP can activitate cytoplasmic streaming and thus induce the movement of chloroplasts. The applicability of this concept on dark-, low- and high intensity orientation movement as well as on induction of centrifugability and cyclosis of chloroplasts is discussed.

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Cytoplasmic streaming within *Paramecium aurelia* II. Cinematographic analysis of the course and reversible cessation of cyclosis [1]

According to Kuźnicki and Sikora 1971, *Paramecium aurelia* immobilized by means of antiserum (Pl. I 1) demonstrates marked cyclosis (Pl. I 2). Contrary to current opinion (Koenuma 1963, Jahn and Bovee 1967, 1969, and Yamada 1969) it was stated that: (a) cyclosis occupies determined regions of the endoplasm (Pl. I 1, 2), (b) velocity of markers (crystals) carried within moving cytoplasm is different in various parts of cell, and (c) a promoting force is localized not only at ecto-endoplasm border.

By means of a long time exposure photographic technique (Kuźnicki and Sikora 1971) it was impossible to determine quantitatively the velocity of separate crystals carried by cytoplasmic streaming along the whole cycle except at a limited distance. To overcome these difficulties, the cyclosis of *Paramecium aurelia* stock 51, syngen 4, serotype A, immobilized by means of antiserum, was recorded under polarized light on 16 mm movie pictures at the rate of 1.6 frames per second on black-white ORWO NP-55 film. Crystals present within *Paramecium aurelia* were chosen as markers of a cyclotic movement of cytoplasm.

Measurement of the velocity of moving crystals was performed by a film analysing projector. The pictures were projected onto a sheet of white tracing paper, the successive positions of a number of separate crystals along the whole cyclosis course were marked and by counting out of frames the time was calculated.

It was generally assumed that cyclosis within *Paramecium* persists continuously if the environmental conditions remain favourable and unchanged. Cessation of cyclosis was observed only under extreme conditions, such as high and low concentration of salts of medium, PCMB (Koenuma 1954, Yamada 1969, 1970). In 22 motion pictures (up to 2 hours each) of the antiserum immobilized *Paramecium aurelia*, four cases were found to manifest a reversible cessation of cyclosis. This phenomenon has never been described by other authors.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

[1] Illustrated by a 16 mm black-white motion picture.

Analysis of motion pictures

The analysis of movement of a number of separate crystals flowing along the whole route without remarkable mechanical stopping (Kuźnicki and Sikora 1972) shows significant differences in velocity of the cytoplasmic streaming. The two successive cycles (Fig. 1) of a single crystal represented as an example. The speed of the crystal ranges from 1.2 to 3.3 μ/sec in the first cycle and from 1.2 to 3.6 μ/sec in the next one (Fig. 2).

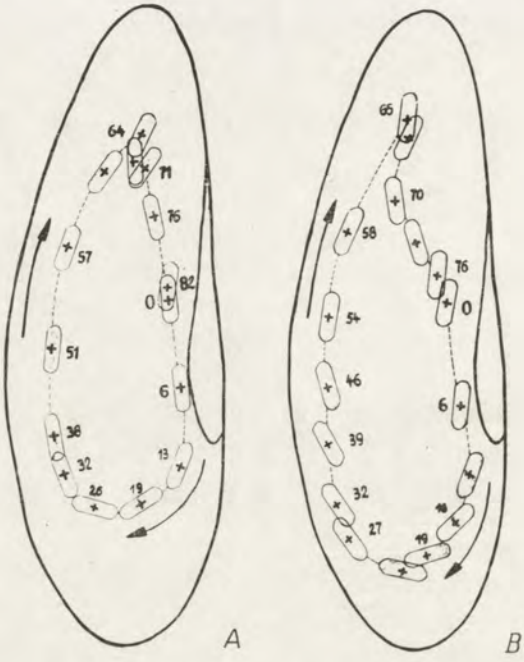

Fig. 1. Two successive cycles (A and B) of single crystal. Numbers show the time (sec) of reaching the successive positions of the crystal. Both cycles are compared in Fig. 2 as a relation of time to velocity of crystal

Starting from the point lying near by a cytostome, the rate of flow decreases and is the lowest at the posterior end of the cell. The velocity of the stream directed anteriorly increases up to the top area where the track of moving cytoplasm turns sharply backwards. At this point the speed of cytoplasmic stream violently decreases. From the top area to the central section of the body the velocity of cytoplasm increases once more.

The paths, velocities and conditions of experiment were comparable to experiments performed before (Kuźnicki and Sikora 1971) and could be regarded as an example of a typical behaviour of cyclosis within the antiserum immobilized *Paramecium aurelia*.

During the time of taking movie-pictures the environmental conditions remained invariable and the ciliates did not exhibit any visible pathological changes. In these conditions, in specimen No. 11 cyclosis stopped twice and started to move again

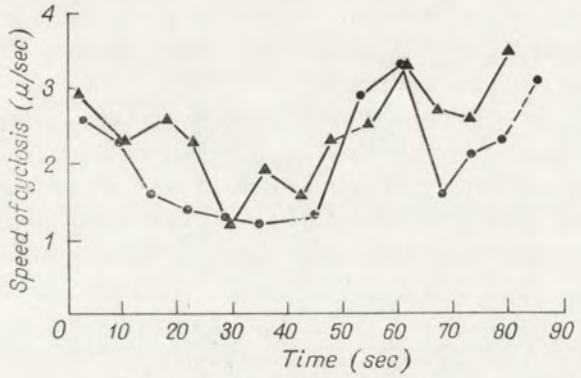

Fig. 2. Speed of separate crystal flowing within cytoplasm stream in two successive cycles, first cycle—circles and second—triangles

in the same pattern, and the mean velocity was the same as before cessation. At first the cessation of cyclosis took place 52 min after treatment by antiserum and persisted for 92 sec. The second cessation occurred 13 min and 30 sec later and persisted for 125 sec, then the cytoplasmic stream started to move again and cyclosis continued the normal course during next 23 min, that is to the end of the film.

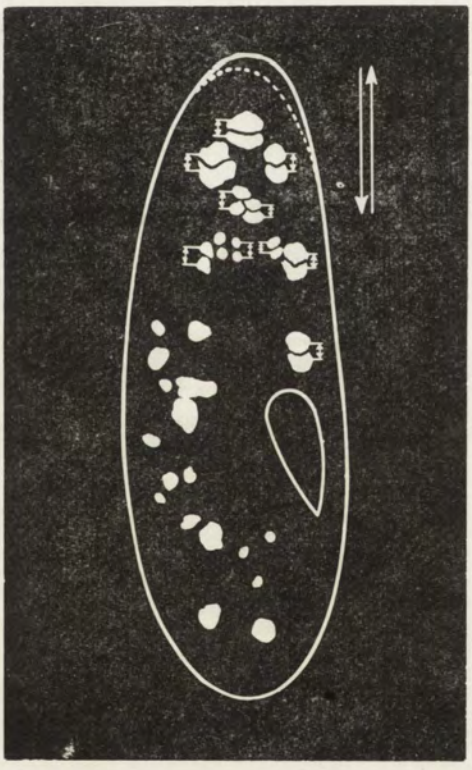

Fig. 3. Reversible contraction of *Paramecium aurelia* body during cyclosis cessation. Positions of crystals and anterior edge marking out the distance and area of contraction

Transition from a normal speed of cytoplasmic stream to motionless state occurred during 8–20 sec. After a complete cessation of cyclosis, usually several ciliary reversals took place one after another at intervals of a few seconds simultaneously with body contractions (Fig. 3), being most strongly expressed in the oral groove. Antiserum immobilized *Paramecium* has cilia sticking together, which makes an impression as if they were stiff and adjusted perpendicularly to the body surface. During ciliary reversal the cilia of those immobilized paramecia only bend forwards from their initial position and after relaxation return to their former position. A transition from one position to the other was synchronous over the whole body surface and was especially strongly expressed in the oral groove cilia.

It should be noted that ciliary reversal is accompanied by a slight contraction of the anterior end of the *Paramecium* body. The contraction evokes shifting of some crystals posteriorly (Fig. 3). When cilia have returned to their initial position, relaxation of the body also occurs and crystals return to their former place without exception (Fig. 3).

The period between the last ciliary reversal and the initiation of cyclosis was never shorter than 12 sec. Cyclosis starts in a less regular pattern, but in the course of a few seconds a complete dynamic organization of cyclosis occurs, similarly as before cessation.

The number of stops of cytoplasmic stream as well as the periods between cessations and duration of cessation vary from specimen to specimen. However, the course of reversible cessation of cyclosis with accompanying ciliary reversal and body contraction is the same for all cases, as it is illustrated in Fig. 4.

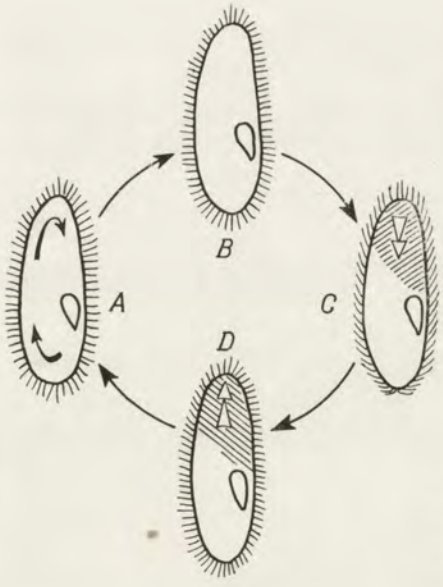

Fig. 4. Behaviour of *P. aurelia* during cessation of cyclosis. From A to B: stopping of cyclosis, from B–C: ciliary reversal accompanied with body contraction, C–D: relaxation, D–A: start to cyclosis movement. Shadowed parts shows contracted area.

Conclusion remarks

Records obtained by means of movie-pictures fully confirmed the conclusions of Kuźnicki and Sikora 1971 that within a *Paramecium* cell cytoplasm flows with different velocities in various parts of the cycle. Moreover, it was possible to measure the velocity of cytoplasm streaming along the whole route of cyclosis.

Except the top area where the track of moving cytoplasm turns sharply back, the velocity of cyclosis corresponds with cross-section of the cytoplasmic stream. The highest rate of movement was noticed in the narrowest area and the lowest one in the widest area. For instance in the regarded case (Fig. 2 B) in position 32, the velocity of crystal was 1.2 μm/sec, and in position 46 it was 2.4 μm/sec. The diameter of flowing cytoplasm in position 32 was 8.4 μm and in position 46 was 3.8 μm. The analysed distance of cytoplasmic streaming is nearly straight and probably circular in cross-section.

In pressure-induced laminar flow of Newtonian liquid the change in velocity when it is passing from a tube of large diameter to one of smaller diameter should fit the classical equation:

$$V_1 = V_2\left(\frac{d_2}{d_1}\right)^2, \tag{1}$$

where V_1 and V_2 represent mean velocities, d_1 and d_2 represent diameters along the large and small diameter sections of the tube. In *Paramecium aurelia* cyclosis, the change of velocity of cytoplasm between wide and narrow area is smaller than expected for pressure-induced flow of liquid. Equation (1) is applicable to the cytoplasmic flow if coefficient k is involved:

$$V_1 = kV_2\left(\frac{d_2}{d_1}\right)^2. \tag{2}$$

In the analysed case coefficient $k = 2.4$ could be calculated because:

$$V_1 = 1.2\ \mu\text{/sec} \qquad d_1 = 8.4\ \mu$$

$$V_2 = 2.4\ \mu\text{/sec} \qquad d_2 = 3.8\ \mu.$$

In other parts of the cytoplasm route of analysed specimen (Fig. 2. A, B) the value of coefficient k varies from 1.4 to 5.0.

Crystals are not the best markers for analysing the velocity distribution across the flowing cytoplasm. However, the plotting of the paths of small crystals by means of film analysis as well as by visual observation of films permitted to observe, that the speed of flow is greatest in the centre of the stream and decreases on the periphery. It is very probable that the velocity distribution across the cytoplasmic stream of *Paramecium*, has much the same profiles as Kamiya and Kuroda 1958 described

in *Physarum polycephalum*. The shape of profile looks like a flattened parabola. This suggestion must be verified by means of other markers.

The most important finding which has been recorded by means of movie-picture was discovering of reversible cessation of cyclosis in *Paramecium aurelia*. This phenomenon was noticed many times not only by means of motion picture. Cyclosis does not seem to be an inherent feature of *Paramecium* remaining in unchanged conditions. Therefore reversible cessation of cyclosis should not be regarded as a casual phenomenon.

At the time of stopping of cyclosis ciliary reversal and body contraction occur. A relation between both kinds of motive activity is unknown. Nevertheless, some indications of the relation between ciliary activity and cytoplasmic movements will be the subject of next paper (Kuźnicki and Sikora 1972).

Summary

By means of motion picture technique cyclosis of *Paramecium aurelia* was studied in specimens immobilized by homologous antiserum. Main features of cyclosis (Kuźnicki and Sikora 1971) were confirmed. The velocity distribution along the whole route of cyclosis is different in various parts of cell and corresponds with cross-section of the cytoplasm streaming. This relation fits the equation of pressure-induced laminar flow of liquid if coefficient k is involved.

Reversible cessation of cyclosis without any change of environment was observed. In the specimen after cessation of cyclosis body contraction probably simultaneous with ciliary reversed occur. Cytoplasm started to move again in a less regular pattern but in short time became regular as before cessation.

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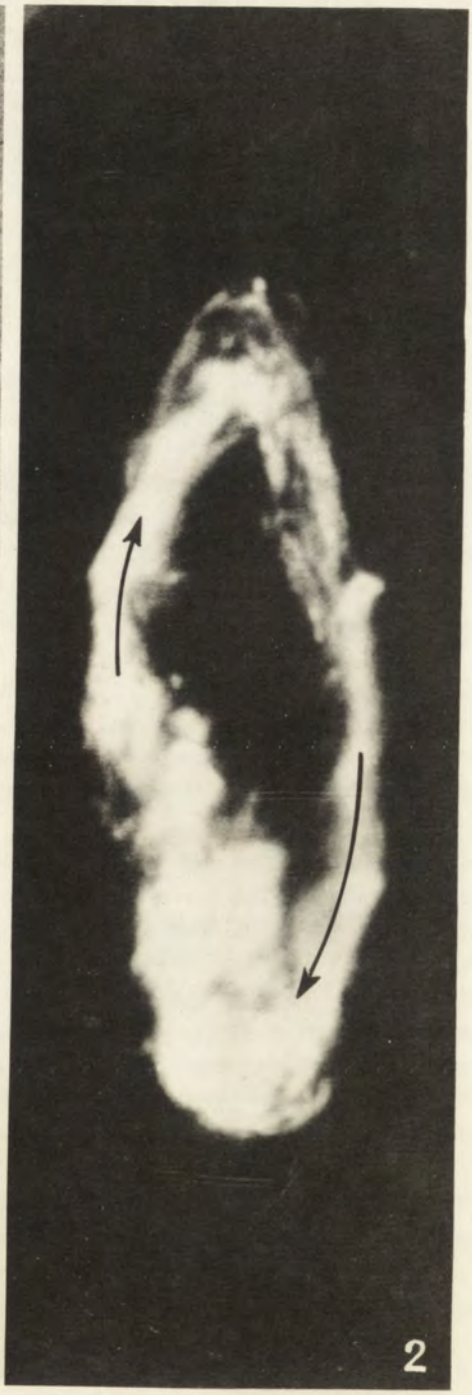
EXPLANATION OF PLATE I

1: Outline of antiserum immobilized *Paramecium aurelia*
2: The same specimen under polarized light in which the shape of the area occupied by cyclosis (moving crystals) is shown. Arrows show direction of cyclosis



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The hypothesis of inverse relation between ciliary activity and cyclosis in *Paramecium* [1]

From the evidence available, it seems that two principal macro-movements of *Paramecium aurelia* cell could be distinguished; ciliary movement and cyclosis. Former investigations were usually concerned with one of those movements alone. The aim of the present study was to find a relation between them.

To characterize cyclosis, the movement of food vacuoles was chosen as a model because of its peculiar properties, such as:

(1) Natural permanent occurrence within cell.

(2) Possibility of defining shape and routes covered within cell.

(3) Possibility of measuring volume and velocity. Some former opinions have to be mentioned about causes and manner of food vacuole movement within *Paramecium* cell.

Ehrenberg 1838 thought that the course of food vacuoles is determined by an invisible for its transparency but permanent digestive tube. He imagined that this tube continue along the cell from the bottom of cytopharynx to the cytopyge. During the second half of XIXth century the idea of existence of a digestive duct in a ciliate cell was finally rejected. The classical drawings of Wallengren 1902, of the character of cytoplasm flow in *Paramecium* (Fig. 1) show that there is no permanent tract of cytoplasm streaming, nevertheless, there are some indications of direction. However, Nirenstein 1905, performed very careful observations on *P. caudatum* and found that food vacuoles in *Paramecium* move along a definite and constant course (Fig. 2 A).

This drawing has been reproduced in Kalmus' 1931 and Wichterman's 1953 monographs. Nirenstein described a small circuit of food vacuole around the macronucleus and a large circuit along the whole cell. The number of small and large circuits varies considerably. To complete circulation the food vacuoles movement lasts from 2 to 4 hours.

The data (Fig. 2 B) obtained by Bills 1922 on the paramecia immobilized by

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Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

[1] Illustrated by a 16 mm colour motion picture.

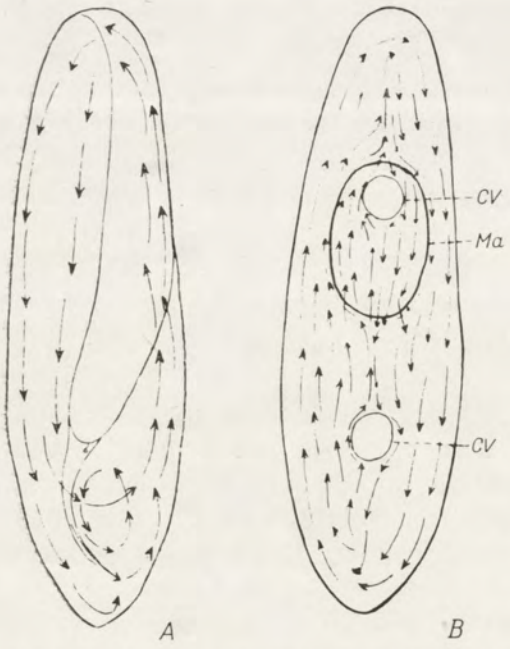

Fig. 1. Course of cytoplasmic movements (indicated by arrows) within *Paramecium caudatum* after Wallengren 1902. The oral view (A) and from the side (B). CV—contractile vacuole, Ma—macronucleus

izopropyl alcohol, correspond quite well with the results obtained by Nirenstein on thigmotactic specimens. Bills has show in his drawings that cytoplasm within *P. caudatum* flows around differentiated regions which correspond with a large circuit described by Nirenstein 1905.

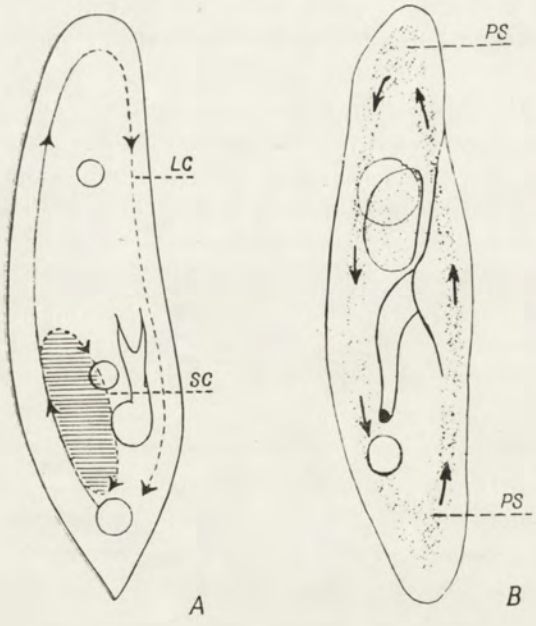

Fig. 2. Cyclosis within *Paramecium caudatum*: A—paths covered by the food vacuole after Nirenstein 1905 (SC—small cycle, LC—large cycle) and B—protoplasmic streaming after Bills 1922 (PS—protoplasmic streaming)

The idea of Nirenstein, Bills and other investigators that food vacuoles are carried by a cytoplasmic stream passively along the same tract was not confirmed by Lubocka and Dembowski 1950. According to their results the routes traced by food vacuoles in the body of *P. caudatum* are extremely variable and do not exhibit any regularity of movement. Lubocka and Dembowski 1950 never observed the large and small circuit of food vacuole.

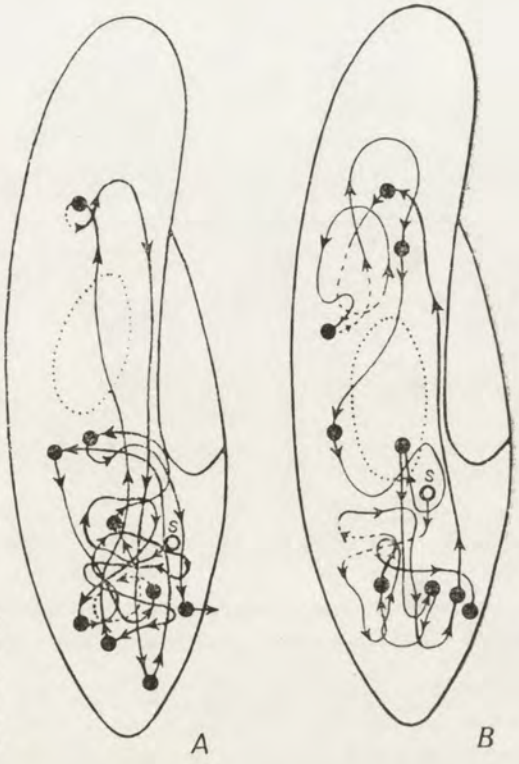

Fig. 3. The routes of food vacuoles of *P. caudatum* containing indigo, after Lubocka and Dembowski 1950, in two different specimens. A—circulation time 200 min and length of route 2329 μm, B—circulation time 150 min and length of route 980 μm, s—starting point

Drawings from Lubocka and Dembowski 1950 paper show the paths covered by food vacuoles containing idigestible (Fig. 3 A, B) and digestible substances (Fig. 4 A, B). No correlation of paths and contents of food vacuole was distinguished. It should be noted that two vacuoles containing the same substances in the same specimen could demonstrate different courses and different length of their routes. According to Lubocka and Dembowski 1950, the total time of the remaining of food vacuoles within the paramecia body and their average speeds, differ in a wide range. For instance, food vacuoles containing indigo move at a speed of 73–108 μm/sec, barium chromate ($BaCrO_4$) 35–135 μm/sec, yeast 30–70 μm/sec, and lettuce 50–206 μm/sec.

According to Lubocka and Dembowski 1950, the routes and velocity of food

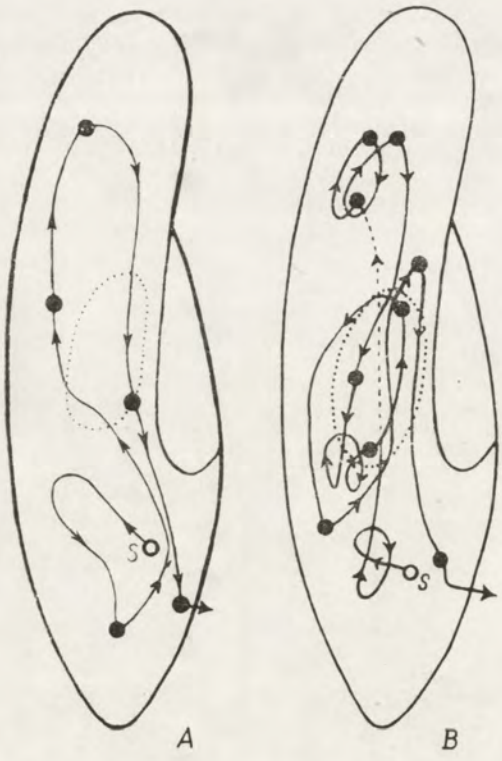

Fig. 4. The routes of food vacuoles of *Paramecium caudatum* containing yeast, after Lubocka and Dembowski 1950, in different specimens. A—circulation time 90 min and length of route 382 μm and B—circulation time 288 min and length of route 544 μm, s—starting point

vacuoles depend on many factors such as: content of vacuoles, the degree of starvation of animals and a number of food vacuoles within, but they failed to give an answer what particular mechanism causes the autonomous movement of food vacuoles.

Cinematographic analysis of food vacuole movement within *P. aurelia*

To solve the problem whether or not the movements of food vacuoles is autonomous, motion pictures of antiserum immobilized paramecia under interference-polarizing microscope were taken. By means of interference technique it was possible to record simultanously the passively flowing crystals and granules and movements of food vacuoles.

Prior to immobilization, the paramecia had been put into suspension of carmine or into suspension of bacteria mixed with a solution of Congo red. *Paramecium* remained in those media for 1 to 5 min, which was a sufficient time to uptake from one to three food vacuoles. Then the specimens were washed in buffer and experi-

mental procedure described previously (Kuźnicki and Sikora 1971) was applied. Cyclosis and movement of food vacuoles were photographed on a 16 mm, UK-18, ORWO-Color film at 1.6 frames per second.

Records collected by means of movie film examined on a film-analyzing projector showed that if the food vacuoles were relatively small (1–3 μm in diameter) they flowed strictly along the same routes as crystals and granules (Kuźnicki and Sikora 1971). However, with an increase of the diameter of food vacuoles there occurred an irregularity of the large circuit. The cause of this irregularity should be mechanical. Large (5–6 μm) food vacuoles very often bung in the narrowest area of cytoplasmic tract, and become obstacles for movement of other food vacuoles. The food vacuoles have the same tendency as crystals to gather at the posterior end of the *Paramecium* body (Kuźnicki and Sikora 1971). In the area where the cytoplasmic stream is widest, the food vacuoles can remain for a long period of time.

The rate of movement of food vacuoles depends on their diameter. If two vacuoles flow along the course of cytoplasmic streaming one after another without any mechanical stopping, their average speeds show an inverse relation to the length of their diameters (Fig. 5 A, B, Pl. I 1–9). The routes and the rate of food vacuoles movements seem to be independent of their contents.

In conclusion, we found no evidence of autonomous movement of the food vacuoles. So they seem to be carried passively by cytoplasmic streaming.

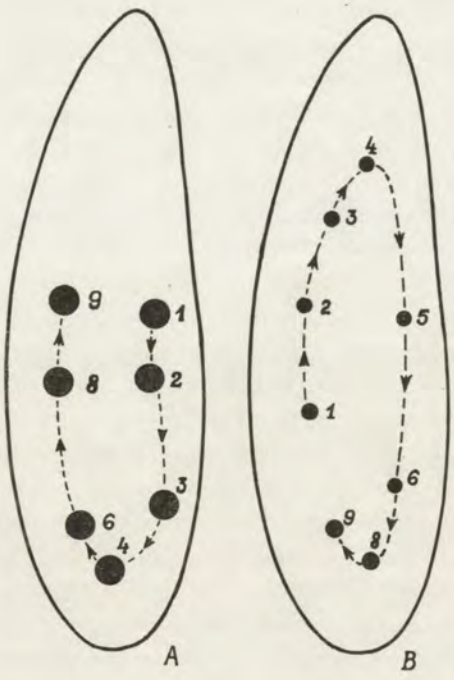

Fig. 5. Successive positions of large (A) and small (B) carmine food vacuoles within the same specimens at the time: 1—0 sec, 2—37.5 sec, 3—105 sec, 4—157 sec, 5—180 sec, 6—210 sec, 7—247 sec, 8—333 sec and 9—378 sec

Discussion

One could think that there is contradiction between the findings of Nirenstein 1905, Lubocka and Dembowski 1950 and present observations. It is possible that in Nirenstein' experiments, paramecia formed relatively small food vacuoles, while in Lubocka and Dembowski's experiments much larger vacuoles were formed. It is well known that the first food vacuole is usually extremely large, especially when uptaken by starved ciliates. We believe that this was the reason of very variable routes of food vacuoles obtained by Lubocka and Dembowski 1950. The small circuit described by Nirenstein 1905 is not common in *P. aurelia*, (Kuźnicki and Sikora 1971) and cannot be considered as a general pattern of cyclosis of food vacuoles.

However, sometimes, the cytoplasmic streamings of opposite sides of *Paramecium* body could be connected by transverse additional stream. In consequence a small circuit of cyclosis (in Nirenstein's 1905 meaning) can take place.

Measurements of the velocity of food vacuoles performed by us along full course have shown that relatively small (up to 3 μm in diameter) food vacuoles flow at a speed of the same order as large crystals. Their mean velocity is about 390 μm/sec, which means their average speed is twice to ten times higher than those noticed by Lubocka and Dembowski 1950 in *P. caudatum*. According to Yamada 1969, the velocity of cyclosis in *P. caudatum* is about twice as high as in *P. aurelia*. So, it seems that differences in the rate of movement of food vacuoles do not depend on a species regarded but rather on external conditions during the course of experiments.

We believe that the rate of cytoplasmic streaming within *Paramecium aurelia*, as well as in other species of *Paramecium* varies widely and depends on motile behaviour of the cell. In Lubocka and Dembowski (1950) experiments cherry glue was applied. This highly viscous medium retarded a swimming rate of the paramecia although during the time of observation the animals were usually moving. In the present experiments, the specimens were completely immobilized. So the assumption is put forth that in *Paramecium*, there occurs an inverse relation between ciliary activity and cyclosis. Some support for this thesis could be found in Yamada's study 1969 on the influence of external tonicities on the speed of cyclosis in four *Paramecium* species. Yamada 1969 wrote: – "When the viscosity of test solutions containing methyl cellulose is not sufficiently efficient to quiet animals, their body cilia often exhibited a transitory vigorous movement. Under such conditions it is observed that the speed of cyclosis is apparently decreased, when ciliary movement stops, the speed reaches maximum". Pilot experiments performed by means of methyl cellulose solutions have shown that specimens do not exhibit visible cyclosis before stopping of ciliary beating. A short-lasting stop of ciliary beat caused by methyl cellulose solution is followed by initiation of cytoplasm streaming.

It should be noted that the results of methyl cellulose experiments confirm previous observations of Kuźnicki and Sikora 1971. 100% specimens treated

by homologous antiserum do not exhibit any cyclotic movement before immobilization, even if they are highly retarded in ciliary movement. The initiation of regular protoplasmic streaming was usually nearly simultaneous with a complete arrest of *Paramecium* swimming movement. Up to now, there have been no sufficient data to prove that only completely immobilized paramecia exhibit regular cytoplasmic streaming, while the swimming ones do not. It should be taken into account that there is a possibility that very slow, and therefore hardly recognizable, cytoplasmic streaming may persist in moving ciliates. Likewise, we agree with Kamiya's 1959 supposition, that cytoplasmic motion is not totally absent on a submicroscopical level in a metabolically active cell.

We believe that the rate of cyclosis is under cell control (Kuźnicki, Sikora and Fabczak 1972) and the relation between ciliary movement and cytoplasmic streaming is of a biological significance. It is well known that paramecia are most active in feeding if they are less active in swimming. When bacteria or other food is added to mass culture, almost all ciliates stop active swimming and exhibit a long-lasting beautiful thigmotactic reaction.

So it seem that there is no doubt about the biological significance of cytoplasmic stream which favours the transport and distribution of uptaken food substances within the cell, particularly at the time of intense feeding.

It is assumed that the rate of cyclosis in motionless paramecia treated by homologous antiserum or hampered by methyl cellulose corresponds closely to cyclotic movement in thigmotactic specimens in culture medium or in other normal conditions.

Contrary to the motionless specimens, in the swimming paramecia cyclosis seem to be stopped at least on the light microscope visibility level.

The hypothesis of the inverse relation between ciliary activity and cyclosis, except Yamada 1969, is essentially different from a generally accepted opinion. For example, Yamashita 1964 wrote: – "In view of the fact that cyclosis continued normally in *Paramecium* when effective ciliary was inhibited or disturbed, it is thought that cyclosis has no direct relation with locomotion or ciliary movement of the animal". This view seems to be doubtful in the light of present data.

A relatively high rate of *Paramecium* swimming makes it impossible to follow the cyclosis within the same time. To overcome this difficulty the paramecia were immobilized by means of isopropyl alcohol by Bills 1922, Hosoi 1937 and Koenuma 1954, 1963. Yamashita 1964 retarded locomotion by methyl cellulose solutions or by nickel sulfate solutions. Yamada 1969 quieted ciliates by methyl cellulose, and Kuźnicki and Sikora 1971 used antiserum. Nirenstein 1905 and Mast 1947 worked on thigmotactic paramecia. Up to now all studies on cyclosis have been limited to the immobilized, hampered or thigmotactic specimens. Nobody has succeeded in making observations of cytoplasmic stream within a swimming *Paramecium*. Therefore, the assumption that cyclosis has no direct relation to locomotion is devoid of evidence. We believe that the inverse relation between ciliary activity and cyclosis is a good example of integration and harmony between two

fundamental motile systems within *Paramecium*. However, till now there has been no technique that would allow direct observations and recording of cyclosis in swimming *Paramecium*. For this reason a working hypothesis, which should be verified, is presented here.

Summary

By means of analysis of motion pictures the movement of food vacuoles within *Paramecium aurelia* was studied. A food vacuole flows along cytoplasmic streaming and pattern of route is comparable with the route of crystals. No evidence was found of autonomous movement of food vacuoles. The rate of cyclosis shows inverse relation to the diameter of food vacuole.

Observations on the behaviour of food vacuoles in swimming specimens suggest that within them cyclosis is highly retarded or even stopped.

The hypothesis of relation between ciliary activity and cyclosis in *Paramecium aurelia* was presented. It was assumed that a swimming animal does not exhibit a visible cyclosis, which could be released after stopping locomotion either by means of immobilizing agents or in the case of thigmotaxis. A number of features of cyclosis and ciliary activity suggest that the proposed relation between them really exists and could be regarded as a manifestation of a control mechanism of intracellular transport.

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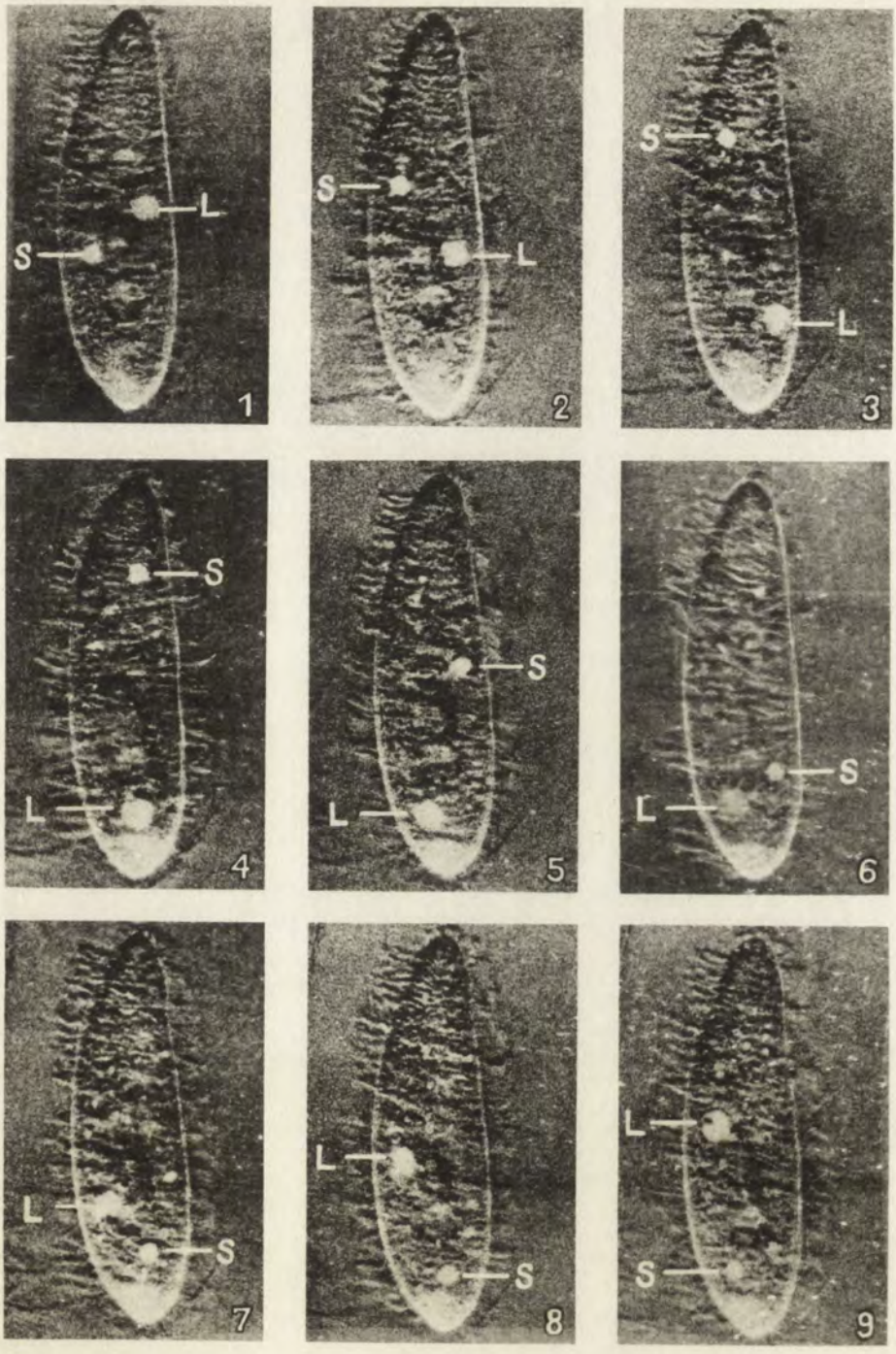
EXPLANATION OF PLATE I

Carmine food vacuoles carried by cyclosis within antiserum immobilized *Paramecium aurelia*. Large (L) and small (S) food vacuoles in successive positions in time: 1—0 sec, 2—37.5 sec, 3—105 sec, 4—157 sec, 5—180 sec, 6—210 sec, 7—247 sec, 8—333 sec and 9—378 sec



L. Kuźnicki et J. Sikora auctores phot.

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Some mechanochemical aspects of flagellar activity

Considerable interest has recently been shown in developing models to explain the conversion of chemical to mechanical energy in biological systems. Models relating to flagellar movement have been developed by Brokaw (1966), Lubliner and Blum (1971) and Miles and Holwill (1971 a). The model of Lubliner and Blum (1971), which develops some of the ideas proposed by Brokaw (1966 a), relies on the attainment of a critical radius of curvature for the initiation of the chemical reaction which bends the flagellum. In their model, Miles and Holwill (1971 a) use the theory of reaction rates to link the chemical event with the mechanical properties of the system. In this paper, the principles underlying the latter model will be briefly reviewed and some alternative interpretations of the results will be presented.

Mechanochemical coupling

The rate of the chemical reactions within a flagellum may be influenced by many factors, such as the temperature and pressure to which the system is exposed. The oscillatory movements of the flagellum will set up stresses within it which may also modify the rate of the internal chemical reactions. In this section equations will be set down to express the relationship between the rate of a chemical reaction and the tension to which the reactants are subjected.

The appropriate equations may be developed from the absolute rate theory (Eyring 1935) in which a reaction is assumed to pass through an unstable transition state (known as the activated state). The rate of reaction is assumed to be determined by the concentration of material in the activated state, a quantity which is known provided equilibrium is assumed between the reactants and the activated state. The equilibrium assumption is not strictly valid, but a comparison with treatments involving non-equilibrium thermodynamics (e. g., Laidler 1965) shows that this assumption incurs little error if the activation energy for the process exceeds 5RT, where R is the gas constant and T the temperature in kelvin. In the case of flagellar acitivity the activation energy is estimated to be about 62 kJ mol^{-1} (Holwill and

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Silvester 1967) which is significantly greater than 12 kJ mol^{-1}, the value of 5RT at temperatures in the region of 20°C.

Since there is some evidence that the chemical reaction which limits the beat frequency of flagella is of first order (Holwill and Silvester 1965, 1967, Holwill 1969, 1970) it is appropriate to discuss the behaviour of a rate constant, k, which characterises a first order reaction.

The absolute rate theory yields

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

where $\Delta G^{\neq}$ is the change in the Gibbs free energy in passing from reactants to the activated state, P is a transmission coefficient (often set equal to unity) while h and $\mathbf{k}$ are the Planck and Boltzmann constants respectively. The free energy change may be expressed in terms of parameters which describe certain physical properties of the system. Of particular interest here is the expression which results when the reactants are subjected to a tension J and undergo a mean change in length $\Delta l^{\neq}$ during activation. It is found that

$$k_J = k_0 \exp\left(\frac{J\Delta l^{\neq}}{RT}\right). \tag{2}$$

where k_J, k_0 are the rate constants at tensions J and zero respectively.

To apply equation (2) it is convenient to specify a model of a flagellum consisting of an elastic filament which resists the bending induced by two bilaterally arranged molecular fibres. The molecular mechanism for bending need not be specified but current ideas favour either a sliding filament system or one in which localised contractions occur.

The form of observed flagellar undulations require that chemical energy be made available at all points along the flagellum (e. g. Holwill 1966). It is convenient to assume that separate chemical reactions are associated with bending and straightening the flagellum at each point. The velocities of each reaction will be given by equations of the same form as equation (2) and may be used to deduce the rate of extension $\frac{de}{dt}$ of an individual molecular fibre. If each bending and straightening reaction is accompanied by a length change of magnitude ε and the fibre has elasticity characterised by a parameter α, we may write

$$\frac{de}{dt} = \alpha \frac{dJ}{dt} + \varepsilon \left[C_S \exp\left(\frac{J_l \Delta l_S^{\neq}}{RT}\right) - C_B \exp\left(\frac{-J_l \Delta l_B^{\neq}}{RT}\right)\right]. \tag{3}$$

In this equation the subscripts S and B refer to the straightening and bending processes while the parameters C are the proportionality constants in the rate equations. J_l is the local tension in the fibre and will in general be a function of the tension

J maintained by the whole fibre and of its effective extension e. Provided J and e are sufficiently small we may write equation (3) in the form

$$\frac{de}{dt}=\alpha\frac{dJ}{dt}+pJ-qe+g, \tag{4}$$

where p, q and g are constants. Equation (4) expresses the mechanochemical coupling which may exist in a flagellum propagating small amplitude waves.

Flagellar wave propagation

Since J and e cannot be directly measured it is necessary to relate these parameters to those characteristics of flagellar waves which can be observed. To do this we consider the following equation developed by Machin (1958) which expresses the behaviour of an elastic beam immersed in a viscous fluid and distorted by an active bending moment M:

$$\frac{\partial^2 M}{\partial x^2}+a\frac{\partial^4 y}{\partial x^4}+b\frac{\partial y}{\partial t}=0. \tag{5}$$

In this equation a is a constant which depends on the elastic properties of the beam while b is proportional to the viscosity of the fluid.

In the model discussed here the active bending moment is supplied by the molecular fibres and will thus be proportional to the tension J, i.e.

$$M=CJ, \tag{6}$$

Where C is a contant. If the distance between the two fibres is $2d$, the extension, e, per unit length of a fibre is given by

$$e=d\frac{\partial^2 y}{\partial x^2}. \tag{7}$$

Equations (4) to (7) can be combined to yield the differential equation

$$(cd+a\alpha)\frac{\partial^5 y}{\partial x^4\,\partial t}+(qcd+ap)\frac{\partial^4 y}{\partial x^4}+b\alpha\frac{\partial^2 y}{\partial t^2}+bp\frac{\partial y}{\partial t}=0. \tag{8}$$

The solution of equation (8) requires the specification of the boundary conditions appropriate to a flagellum. These are not known for real flagella, but it may be shown that progressive waves of sustained, finite amplitude are only possible if the transverse and angular impedances at the ends of the flagellum are neither zero nor infinite. A solution corresponding to a progressive wave of finite amplitude shows that the wavelength $\left(\frac{2\pi}{k}\right)$ and frequency $\left(\frac{\omega}{2\pi}\right)$ of the oscillation are given by

$$pb+k^4(cd+a\alpha)=0 \tag{9}$$

and

$$\omega^2=-\frac{p}{\alpha}\left(\frac{ap+qcd}{cd+a\alpha}\right). \tag{10}$$

Discussion

In a previous paper (Miles and Holwill 1971 a) it was shown that reasonable values for the physical and chemical parameters in equations (9) and (10) could lead to values of k and ω which are typical of flagellar waves. Equation (9) and (10) can be manipulated to describe the behaviour of the frequency and wavelength as functions of a number of physical and chemical variables. In particular the effects of viscosity and temperature on intact organisms and of ATP concentration on glycerol extracted flagella may be predicted. Satisfactory agreement between the theory and experimental results is found for a number of organisms (Miles and Holwill 1971 b) and the graphs obtained allow estimations to be made of the mechanochemical parameters in equations (9) and (10).

In the earlier papers (Miles and Holwill 1971 a, b) the prediction of equation (10) that the frequency does not vary with the external visocsity was cited as a failure of the theory, and it was suggested that further terms were needed in the differential equation (8) to completely describe the system. An alternative view is that the effects of viscosity are completely described only when both equations (9) and (10) are considered. By eliminating $(cd+a\alpha)$ between these equations the following relationship may be obtained after some rearrangement:

$$V_w^2\lambda^2=(2\pi)^2\frac{(ap+qcd)}{\alpha b}, \tag{11}$$

where V_w is the wave velocity and λ the wavelength. This interpretation is not unreasonable when the experimental results are considered, for a change in the viscosity influences not only the frequency of beating but also the wavelength of the undulation. Since b is directly proportional to the viscosity of the medium, equation (11) predicts that a plot of $V_w\lambda^2$ against $1/\mu$ should be linear. Figure 1 is an example of this plot for *Crithidia oncopelti*, using data published by Holwill (1965). Similiar linear plots are obtained for a number of invertebrate spermatozoa using data from Brokaw (1966 b). The linear relationship obtained for *Crithidia oncopelti* is encouraging, since this organism has yielded data which have been difficult to predict from the behaviour of model systems (e.g. Miles and Holwill 1971 b, Lubliner and Blum 1971).

It thus appears that equation (11) should be used to investigate the model system

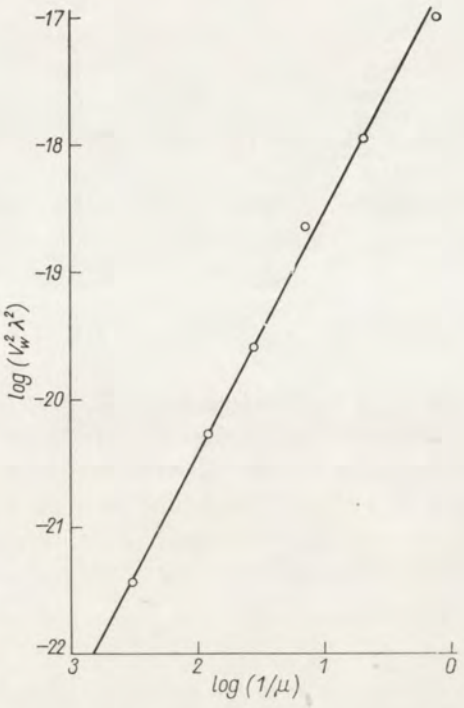

Fig. 1. The variation of flagellar wave velocity (V_w) and wavelength (λ) with viscosity (μ) for the organism *Crithida oncopelti*. (Data from Holwill 1965)

for comparison with the behaviour of real flagella. In a previous paper (Miles and Holwill 1971 b) equation (9) and (10) were, in general, used separately for this purpose. In particular, equation (10) was used to predict the variation of beat frequency with temperature and ATP concentration (for glycerinated organisms). The good agreement which was found between the predictions of equation (10) and the experimental results would also exist for equation (11), since the parameters of equation (9) are rather insensitive to changes in temperature and chemical environment.

While the mechanochemical model summarised above appears to be satisfactory in many experimental situations, the model itself is limited to small amplitude waves. The waves observed on real flagella are of large amplitude, and it would be useful to relate the magnitude of this parameter to the mechanochemical event within the flagellum. A possible method for doing this may be in the development of the rate equation (1) to include terms for the internal and external work which is performed by or on the flagellum.

Following Holwill and Silvester (1965) the rate constant k is assumed to be proportional to the flagellar beat frequency. Equation (1) may then be rewritten as

$$f=\zeta \exp\left(\frac{\Delta W_i-\Delta W_e}{RT}\right), \tag{12}$$

where ζ is a constant and ΔW_i, ΔW_e are changes in the internal and external work. The amount of work performed externally by the flagellum can be calculated if the waveform is given, since the hydrodynamic theory relating to the viscous interaction between the flagellum and medium is reasonably well established (e.g. Holwill 1966). The internal work done by the flagellum is not easy to calculate, since the elastic properties of the system are not well established. However, by assuming equation (12) some indication of the internal work can be obtained from a study of the effects of viscosity on flagellar beating. Equation (12) may be rewritten

$$\ln f = \ln \zeta - \frac{\Delta W_i}{RT} - \frac{\Delta W_e}{RT} . \tag{13}$$

If ΔW_e is much larger than ΔW_i or ΔW_i remains constant, a plot of $\ln f$ against external work will be linear. Figure 2 shows such a plot for spermatozoa from *Chaetopterus* using data published by Brokaw (1966 b). For large values of the external work, the plot is linear but becomes curved at lower values. A possible interpretation is that the difference between the curved region and the projection of the linear

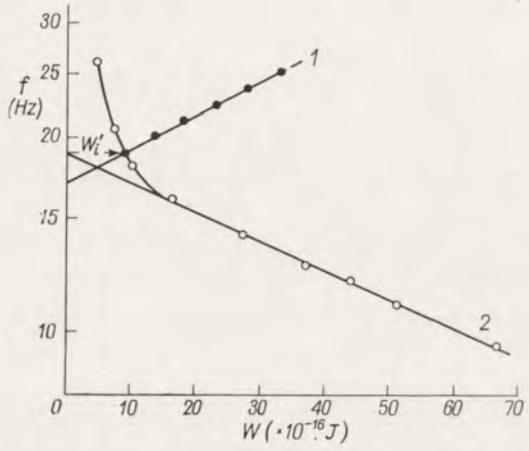

Fig. 2. Semi-logarithmic plot showing the variation of work per flagellar beat (W) with beat frequency (f) for spermatozoa from *Chaetopterus variopedatus*. 1—internal work (W_i) and 2—external work. (Data from Brokaw 1966 b)

portion (e.g. W_i in Fig. 2) represents the internal work increment. A graph of internal work obtained in this way against the logarithm of the frequency is also linear (Fig. 2), a property which may be attributed to the fact that the internal work in this region is significantly larger than the external work. It is interesting to note that when the same procedure is followed for the organism *Crithidia oncopelti* the relative internal work is found to be directly proportional to the log of the frequency, but the slope

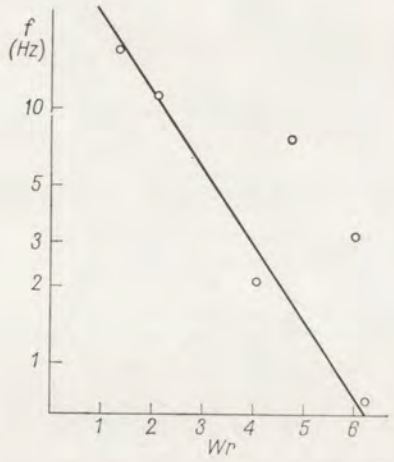

Fig. 3. Semi-logarithmic plot showing the variation of relative internal work (W_r) with frequency (f) for the organism *Crithidia oncopelti*

of the line is opposite to that for *Chaetopterus* (Fig. 3). The significance of these results is not clear, but further development of these ideas may lead to useful concepts when dealing with large amplitude flagellar waves.

Summary

A model in which the biochemical and mechanical approaches to flagellar activity are unified is discussed. Published experimental results are found to agree with the predictions of the model. Thermodynamic equations are derived from which it may be possible to obtain information about the internal properties of flagella.

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Effects of laser irradiation on the structure and function of cilia and flagella

A number of mathematical and physical models of flagellar beating have been proposed and continue to be proposed. There has, however, been relatively little experimental testing of these ideas. The results of experiments involving damage of small regions along flagella can be interpreted in terms of some predictions of these models. In addition, the technique has been used in the study of differences of structure and function among flagella.

In these experiments, small regions of actively beating intact flagella were irradiated with a pulse from a ruby laser microbeam. The functional effects of the resulting damage were recorded photographically. The effects of this irradiation on the ultrastructure of cilia have been examined with the electron microscope.

Material and methods

Experiments were performed on flagella of echinoderm spermatozoa, and the results were recorded on dark-field multiple-exposure plates (Goldstein 1969). They were also performed on flagella of a trypanosomatid flagellate, *Crithidia oncopelti*, and the results were recorded on high-speed cine film (Goldstein, Holwill and Silvester 1970). The laser emitted 694.3 nm light in pulses of approximately 250 μs duration. Blue dye dissolved in the water absorbed the radiation. The irradiated region was nominally approximately 2 μm across. Degree of damage ranged from very slight to complete slicing of a flagellum.

Electron microscopic examination of the effects of laser irradiation on ciliary ultrastructure was carried out on the lateral gill cilia of a fresh water mussel, *Elliptio*. Large lesions (approximately 30 μm in diameter) were made in actively beating tissue, and the tissue was prepared for electron microscopy. The tissue was embedded in a flat sheet. Areas containing lesions were mounted on blocks and sectioned. Cilia with varying degrees of damage could be found in the periphery of a lesion (Goldstein and Satir 1971).

Results

Echinoderm spermatozoa

In these flagella, bends originate at the base and travel to the tip.

The region between the irradiated point and the head could beat for at least

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

a few periods if the region was greater than about 10 μm in length (Pl. I 1). In the region beyond the irradiated point, bends which were already established at the time of irradiation continued to travel to the tip (Pl. I 2). The velocity of propagation along the flagellum decreased, and the size and shape of a bend often changed. No new bends ever formed beyond the irradiated region, so that this region was straight after the old bends had travelled off the tip (Pl. I 1).

Irradiation in the center of a bent region apparently caused that region to straighten (Pl. I 3).

The results are described in detail in Goldstein 1969.

Flagella of *Crithidia*

In these flagella, bends can originate at either the base or the tip (Holwill 1965). They usually beat from the tip, and commonly reverse the direction of beating.

When bends were originating at the base, they continued to do so after irradiation of the tip, and they would begin to originate at the tip after irradiation of the base. When bends were originating at the tip, they usually continued to do so after irradiation of the base, and they began to originate at the base after irradiation of the tip. Occasionally, however, bends would originate at or near the irradiated region and travel toward the tip. This could happen even in fragments which were completely severed from the cell body (Pl. II 4, 5, 6).

After irradiation, isolated fragments could beat for up to ten cycles. The cell bodies often disintegrated within about a minute.

These results are described in detail in Goldstein, Holwill and Silvester 1970.

Electron microscopy

Cilia appear coagulated in an irradiated region. The matrix, which is difficult to preserve under the best of conditions, was very easily damaged, and has a mottled appearance (Pl. II 7). Both the central pair and the outer doublets were affected, but the central pair was noticeably more sensitive than the outer doublets (Pl. II 8). In this regard, these results are similar to those under conditions of chemical dissection and negative staining: the central pair also decomposes more easily during these procedures. The doublets sometimes appear to fuse. The membrane apparently sealed over the stumps of severed cilia (Pl. II 9).

The refinement of these techniques to the point of electron microscopic examination of individually irradiated cells might allow comparison of ultrastructural damage with corresponding effects on motility.

Discussion

In flagella of echinoderm spermatozoa, as in most flagella, bends always originate at the basal end. In contrast, they may begin at either end in *Crithidia* flagella. The

flagella of both echinoderm spermatozoa (Afzelius 1955) and *Crithidia* (Burnasheva, Ostrovskaya and Yurzina 1968) have a simple 9+2 ultrastructure. The irradiation of these two types of flagella provides a means of testing some predictions of different models of flagellar motility.

Two classes of mechanical models have been proposed. In one (Machin 1958, 1963), the entire flagellum beats as though it were a set of coupled oscillators, any one of which is potentially capable of initiating bends. In the other (Brokaw 1966), only the basal end (and the tip in cases such as *Crithidia*) is capable of initiating bends; other points along a flagellum bend only when triggered by bending at adjacent points.

When an echinoderm sperm flagellum was irradiated, a portion could beat only if it contained the basal end. The lack of bend initiation distal to an irradiated region was not due simply to depletion of ATP in that region (Goldstein 1969). In these flagella, then, the ability to initiate bends appears to be localized to the base, as predicted by the model of Brokaw (1966).

When a *Crithidia* flagellum was irradiated, bends could continue to originate at the intact base or tip. In addition, bends could originate at or near the irradiated region and pass to the tip, even when the flagellum was severed near its center. This indicates that, under some conditions, bends can originate at points other than the original base and tip. They thus appear at first sight to behave as predicted by the model of Machin 1958, 1963. There is one way, however, in which their behaviour seems to differ from that predicted by this model. The model predicts that beating should begin as a standing wave which develops into a travelling wave as the amplitude increases. But bends appear to start at one end as a travelling wave, without a preliminary standing wave. This is true even at the new "end" of an isolated fragment. These results might mean that the model is formally correct, but that its parameters describe characteristics other than gross bending.

The behavior of irradiated *Crithidia* might also fit a modified version of the model of Brokaw (1966). Since bends originate only at an end, the properties of this flagellum may be due to the ability of any point to initiate bends if and only if it is on an end. Bends could be initiated by end regions, and just propagated at interior points. Perhaps some flagella will be found which are normally capable of initiating bends only at their base, but which are capable of initiating bends at other points after amputation.

Beating in isolated fragments indicates that the complex basal structures are not necessary for beating. There are other indications of this: several types of cilia and flagella which have been removed from a cell – and apparently from their basal body – have been able to beat in the presence of ATP, and some invertebrate spermatozoa which apparently lack a basal plate exhibit typical flagellar beating.

The ability of isolated fragments to reverse the direction of bend propagation indicates that this behavior is an inherent property of the flagellum of *Crithidia*.

The apparent disappearance of a bent region following irradiation of the center

of that region in echinoderm flagella suggests that an entire bend is somehow maintained and propagated as a unit. The possibility of integration of bending within a bent region has so far received little attention in theoretical models, and invites further exploration.

These experiments indjcate well-defined differences between the responses of two types of flagella to laser-induced lesions. These results suggest refinements in present models of flagellar motility.

Acknowledgements

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The irradiation studies on *Elliptio* were supported by USPHS grants HE 13849 and FR 7006-05.

Summary

Actively beating flagella of echinoderm spermatozoa and of *Crithidia oncopelti* were irradiated with a single pulse from a ruby laser microbeam. The results were recorded photographically. Lateral cilia of the gill of *Elliptio* were similarly irradiated, and the irradiated cilia were examined by electron microscopy.

The matrix of irradiated regions has a mottled, coagulated appearance. The matrix and central pair are more easily damaged than the outer doublets.

Flagella of echinoderm spermatozoa apparently require their basal end for motility, while those of *C. oncopelti* apparently need neither their base nor their tip.

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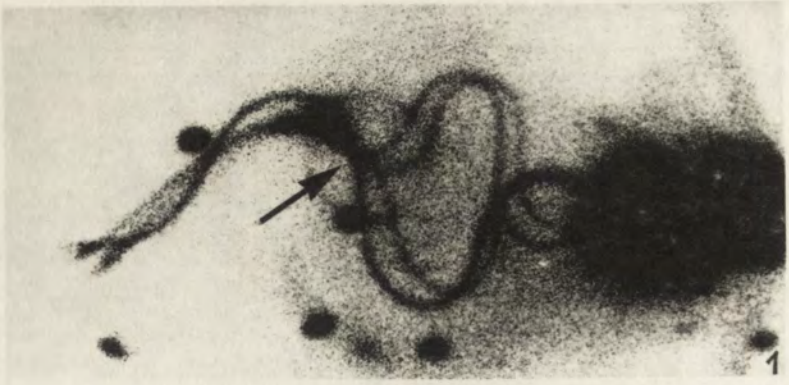
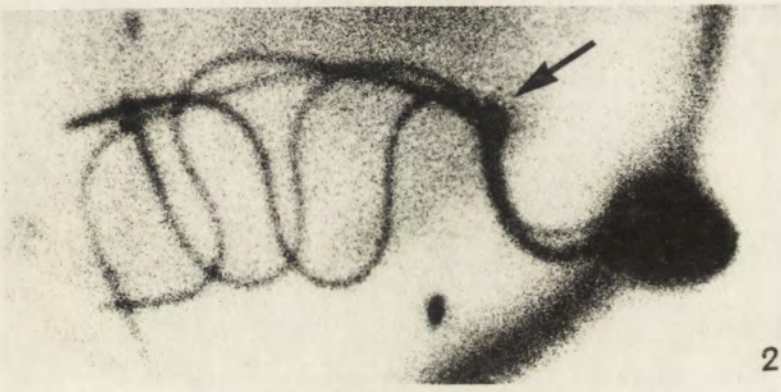
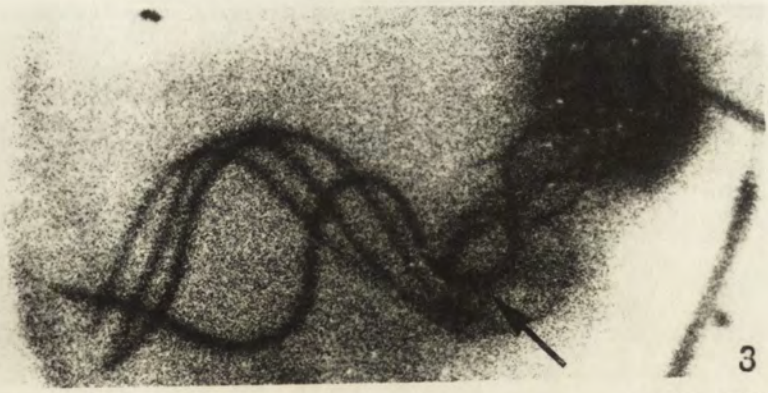
EXPLANATION OF PLATES I–II

1: Echinoderm spermatozoon beating between irradiated point (arrow) and head
2: Bends distal to irradiated point (arrow) continue to travel to the tip
3: Irradiation in center of a bent region (arrow). That bend does not appear on subsequent exposures
4: Flagellum of *Crithidia* immediately after being severed at the base. Waves were originating at the tip
5–6: Same flagellum as 4, approximately 800–900 ms later. Time between 5 and 6 is approximately 70 ms. A bend (arrows) is shown travelling toward the tip
7: Irradiated flagellum (arrow) showing mottled appearance of matrix. Marker is 0.2 μm.
8: Irradiated flagella (arrows), showing greater destruction of central pair than of outer doublets. Marker is 0.2 μm
9: Irradiated flagellum (arrow) showing apparently intact membrane sealing over the irradiated region. Marker is 0.2 μm







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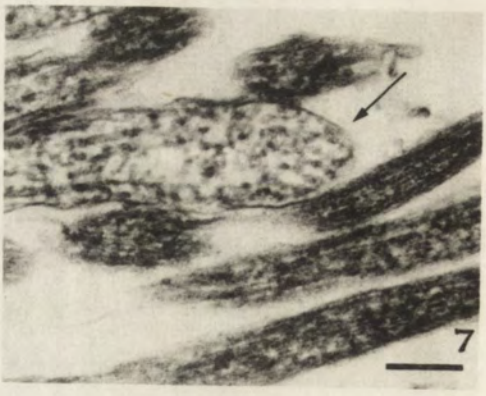

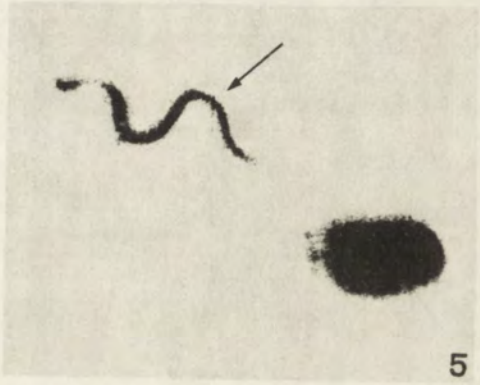

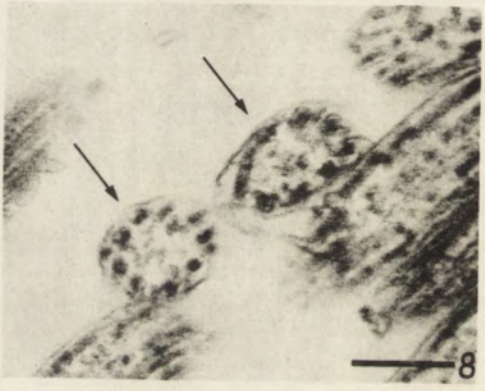

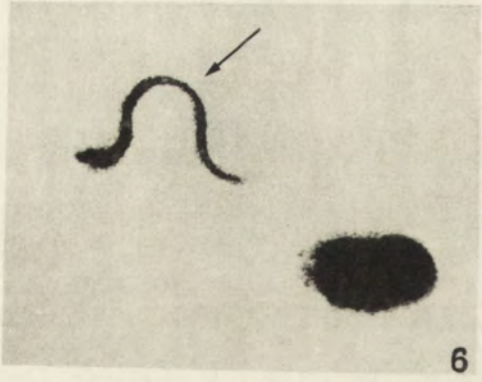

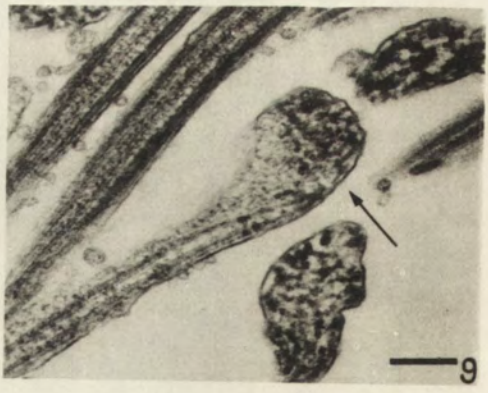

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The movement of water by cilia

There are one or two descriptions in the literature of the pattern of movement of water around moving flagella, e.g. by Lapage (1925) for *Codonosiga* and by Jahn, Harmon and Landman (1963) for *Ceratium*, and some data on the rate of swimming of spermatozoa and flagellates (see Holwill 1966). As far as cilia are concerned, however, there is a little published information on the flow of water caused by cilia (see e.g. Machemer 1966), but no quantitative data on the relationship between the rate of flow of water produced by a cilium and the movement of the cilium itself. In this paper we have described the pattern of movement of water around moving cilia of three types, and compared the rate of movement of the water with the rate of movement of the cilia. One important finding of this study is that the detailed form of the metachronism shown by the cilia is intimately interrelated with the activity of the cilia in the maintenance of water flow, so that it will be necessary to describe the motion and the metachronism of the cilia in each example as well as the motion of water that is caused by the activity of the cilia.

Materials and methods

The three examples studied were the membranelles of the ciliate *Stentor polymorphus*, which is maintained in culture (Sleigh 1956), the comb plates of the ctenophore *Pleurobrachia pileus*, obtained from plankton in the English Channel and the lateral cilia on gill filaments of *Mytilus edulis* obtained from the shores of the English Channel. The motion of the cilia was studied by direct microscopical examination, using various optical techniques including Nomarski interference, and quantitative data on the movement of the cilia was obtained by the use of stroboscopes and the analysis of high-speed cine films. The movement of water around the cilia was followed by tracing the movements of particles (usually 2.7 μm diameter polyvinyltoluene latex particles (Dow)) from frame-by-frame analyses of cine films of the active cilia taken at speeds of between 16 and 330 frames per second. All observations recorded here were made at temperatures of 20 to 22°C.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Results

Membranelles of *Stentor*

The flow of water around an attached, feeding *Stentor* is maintained principally by the beating of the membranelles which occur at intervals of 3–4 μm in a single row around the broad oral end of the animal. Each membranelle is about 25 μm long and is a compound structure consisting of some 60 to 75 cilia which move together as a single unit. The membranelles are extended "upwards" at the beginning of the effective stroke of the beat; during the effective stroke the compound cilium bends in the basal region and the shaft of the membranelle swings outwards and downwards in a plane approximately at right angles to the row, the tip of the membranelle moving through a distance of about 40 μm in about 9 ms at an average tip velocity of about 4400 μm/s. The bend which forms at the base of the membranelle during the effective stroke then passes up the membranelle and is followed by a straightening which completes the recovery stroke of the beat and returns the membranelle to the starting position (Sleigh 1968). The whole cycle of beat normally occupies 25–40 ms at about 20°C. The activity of the membranelle is coordinated into metachronal waves which pass along the row of membranelles from one end to the other (Plate I 1), the interval between the beginning of the effective stroke of one membranelle and that of the immediately following one being between 5 and 6 ms.

This ciliary activity causes a flow of water from in front of the body backwards and towards the sides, so that a circulating vortex is formed all around the edge of the peristome. Cine films taken at 16 frames/s show that some flow of particles occurs as much as 0.5 mm away from the active membranelles. The flow towards the peristome accelerates as it narrows near the row of membranelles, and the particles which approach the body are either caught on the peristomial field within the ring of membranelles or pass outwards to the side where they may move down the side of the body or enter a circulating vortex current (Fig. 1). In such a view of the body one can only see the form of the vortex clearly at the side of the body, and only in this region can one see adequately the relationship between the rate of flow of water and the movement of the membranelles. The vortex has a centre about 70 μm from the bases of the membranelles, and the outer side of the vortex (where the water moves slowly) extends several hundred μm from the edge of the body. The fastest flow of particles occurs in the region of the tips of the membranelles, where speeds of up to 800 μm/s have been measured.

In spite of the fact that a membranelle is moving back against the water flow (in the recovery stroke) for about 2/3 of the cycle of beating, the forward flow of water appears quite smooth and continuous, even in films taken at 330 frames/s. When one membranelle performs its effective stroke, the tip moves forward at a speed of about 4400 μm/s, and before this membranelle has completed its swing the neighbouring membranelle, at a distance of only 3–4 μm, commences to move forwards

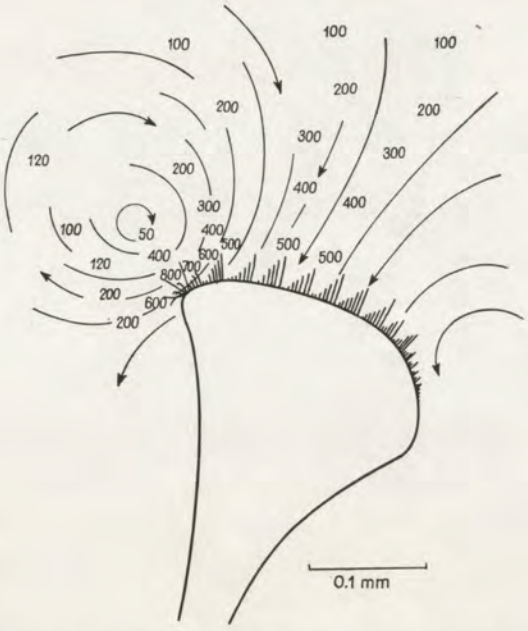

Fig. 1. Composite diagram from analysis of cine films showing the paths taken by particles in the water around a feeding *Stentor*; the numbers indicate the average speed of particle movement in μm/s

in a parallel plane, to be followed after a similar interval by the next, and so on. Although the system functions at a low Reynolds number, so that the relation between driving force and water movement is governed by viscosity rather than inertia, the water flow is maintained continuously because of the cooperative activity that is the main result of the metachrony of the membranelles. At any instant there are always some membranelles driving water forwards and some membranelles recovering, and no part of the adjacent water current is ever very far from a membranelle that is propelling water during its effective stroke.

The maximum speed of water flow is observed at a level near the tip of the membranelles and the rate of flow decreases nearer the body and at a greater distance. This is shown in Fig. 2, where the distance moved by a particle during one cycle of beat of a membranelle is compared with the distance of the particle from the base of the membranelle. It is important to notice that perhaps one half of the water moved by the membranelles is carried forwards by viscous drag in the region beyond the tips of the membranelles, outside the area through which the cilia move. The maximum speed of flow measured was only about one quarter of the tip velocity of the membranelle, but there was detectable forward flow at a distance from the body surface equal to almost three times the length of the membranelles.

Comb plates of *Pleurobrachia*

The relationship between the movement of comb plates and the movement of water is different from that in *Stentor* because here the plates move in succession in a single plane rather than in parallel planes. The comb plates of such regular-shaped

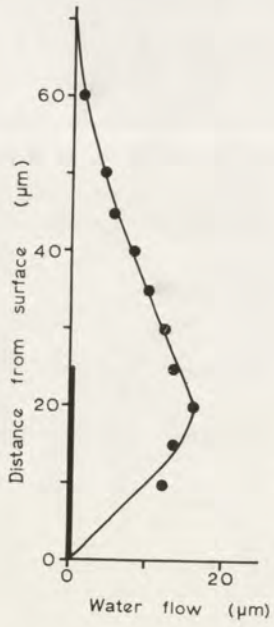

Fig. 2. The relation between the distance that particles are carried in the water flow during one cycle of beat of a membranelle of *Stentor* and the distance of the particles from the base of the membranelle along a line drawn between the membranelle base and the centre of the vortex. The length of a membranelle is indicated on the ordinate axis

ctenophores as *Pleurobrachia* are arranged in eight rows (Fig. 3). Each plate is a flat rectangular paddle, made up of many thousands of cilia arranged in 20 or more rows aligned in the plane of the paddle; those shown in Figs. 4 and 5 are about 800 μm wide and 600 μm long. The whole plate normally moves as a single unit in very much the same manner as a single cilium, with characteristic effective and recovery strokes (Sleigh 1972). In the effective stroke of the beat the plate swings towards the aboral end of the animal, so that it normally swims mouth foremost. The effective stroke is a rapid swing of the extended plate, while in the recovery stroke the bent plate moves back more slowly to the starting position, passing through a smaller volume of water. Comb plates may be driven to beat at different frequencies under identical conditions of temperature etc., and at a low frequency the effective stroke and recovery stroke are both similar in shape to those at a high frequency, but both take place more slowly (Sleigh 1972). Metachronal waves of beating set out from the aboral end of each comb row and pass down the row in the direction opposite to the effective stroke of the beat, involving each plate in turn.

The movement of comb plates and the movement of the water around them have been analysed from films taken at 330 frames/s (Plate I 2). Information on two examples is given here, one where the comb plates were driven to beat at about 5 cycles/s (Fig. 4) and the other where they were driven at 12 cycles/s (Fig. 5). The whole cycle of beating is made up of several distinct phases in which the movement of water shows different features, and so the cycle has been broken up into several stages in order to display the information more clearly. The position of the comb plates is

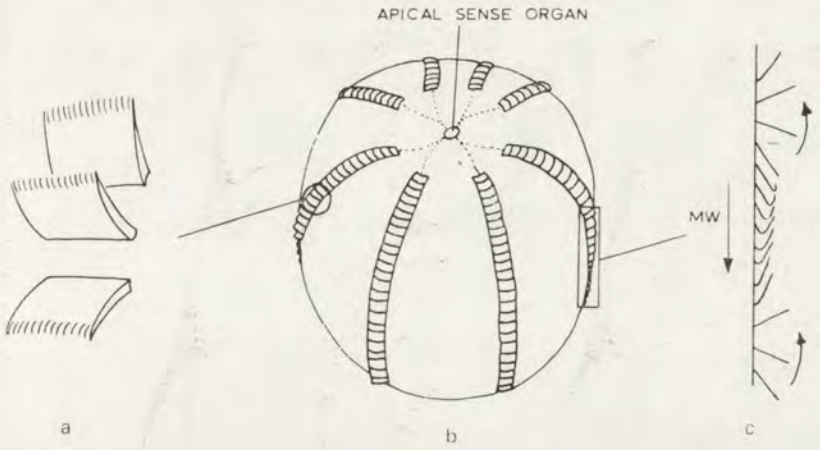

Fig. 3. Diagrams showing (a) the shape of comb plates of *Pleurobrachia*, (b) the arrangement of these plates on the body and (c) a profile view of a row of comb plates showing the relationship between the downward movement of the metachronal waves (MW) and the upward active strokes of the individual plates

indicated at intervals of 10 ms by a succession of profiles, and the position of particles at the same times by a succession of symbols.

At the beginning of a cycle of beat (Fig. 4 a, 0 ms) the comb plate at the right begins to beat first; as it swings upwards and to the right at the start of its effective stroke, water is drawn into the space formed between this plate and the centre one, the water entering the space from above, from behind and from the sides (although the last is not shown in this Figure). Once the second plate begins to move, after almost 10 ms, an approximately constant angle is maintained between the first and second plates, so that a body of water is carried to the right between the two plates; at the same time water is drawn in behind the second plate and is then carried to the right between the second and third plates. Towards the end of its effective stroke the first plate begins to slow down (30–40 ms) and water is squeezed out to the right from between the first and second plates as the second plate approaches the first; this squeezing out is perhaps seen even more clearly between second and third plates at 40–60 ms (Fig. 4 b). The water propelled to the right during the latter part of the effective stroke continues to flow over plates that are engaged in their recovery strokes, so that a vigorous flow of water is maintained throughout the period shown in Fig. 4 c, in spite of the fact that none of the plates shown is concerned with driving the water in the forward direction; in fact all are moving back against the flow of water, but beneath it. The forward flow of water continues as the plates rest between beats (140–170 ms, Fig. 4 d), but by the time the first cilium begins to move forwards again at 180 ms, the flow has almost stopped, only to accelerate again as the next cycle of beat commences. The tips of the plates move forwards at about 50 mm/s

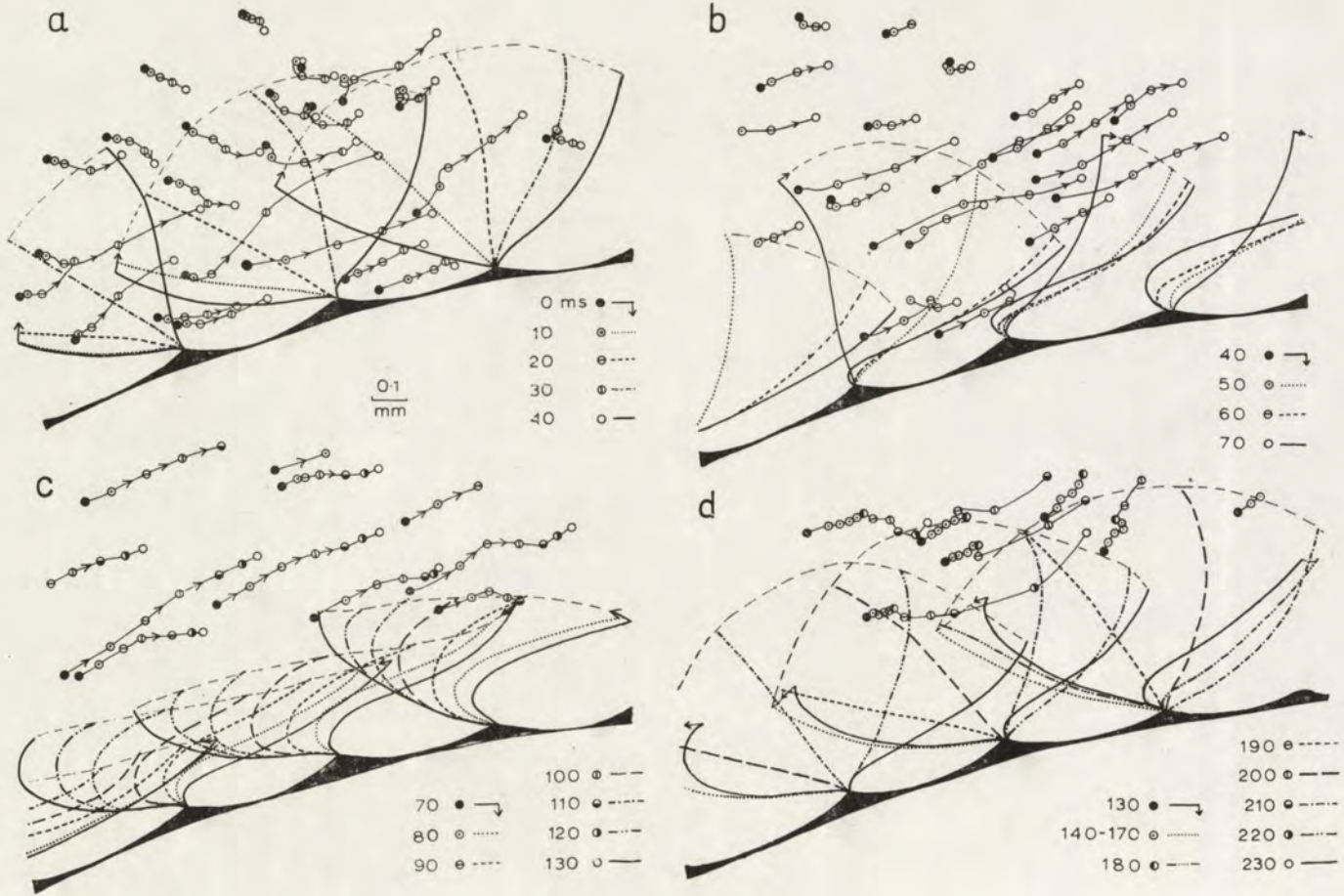

Fig. 4. The shape and positions of comb plates of *Pleurobrachia*, as seen from one side of the comb row, at intervals of 10 ms during 230 ms for a row of plates beating a little over 5 times/s; in each part of the figure the profiles of comb plates at different times are shown by the forms of line in the lower right corner of the part. The positions of selected particles suspended in the water around the comb plates at the same time intervals are shown by the symbols indicated on each part of the figure. The movements of comb plates and particles are described in the text

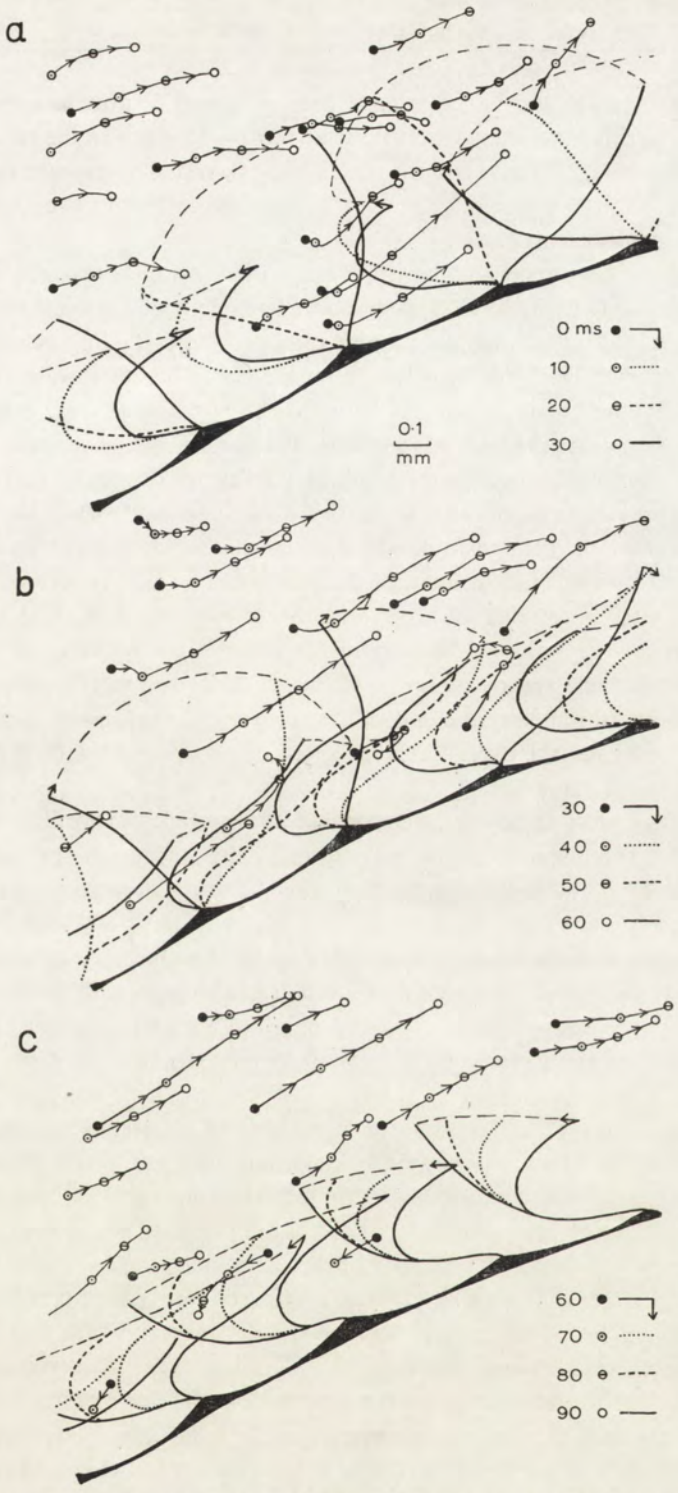

Fig. 5. As Fig. 4, but with profiles of comb plates and positions of particles at intervals of 10 ms during 90 ms for a row of plates beating at about 12 cycles/s

in the effective stroke, and particles have been observed to flow at speeds of up to 30 mm/s during this time; the particle flow falls to 10–15 mm/s in the recovery stroke and decreases almost to zero after the plates come to rest. The average flow throughout the beat is about 6 mm/s, but this is of course the result of the coordinated activity of a number of comb plates.

In the cycle of beating at a higher frequency (Fig. 5) similar features may be seen, the water being drawn in behind a rising plate, being carried between adjacent plates and being squeezed out as one plate moves closer to the preceding one at the end of the effective stroke. The velocity of water movement is higher throughout the beat, as indicated by the greater distances between the symbols in Fig. 5 as compared with those in Fig. 4. At the beginning of one effective stroke the water is still moving rapidly as a result of the activity of comb plates further down the row that are involved in the previous metachronal wave; this flow is especially obvious in the region above the zone swept through by the tips of the plates. During the recovery stroke the fairly rapid backward movement of the plates can be clearly seen to carry water between the plates in the direction opposite to the effective flow (50–90 ms in Figs. 5b and c). In this case the tip velocity of the plate in the effective stroke is about 70 mm/s, and particle speeds of up to 40 mm/s occur during the effective stroke, slowing to about 10 mm/s by the end of the recovery stroke; in this case there is no rest between beats and the overall average speed of particles at the level of the ciliary tips is about 16–18 mm/s.

It is again interesting to plot the distance the particles move during one cycle of beat against the distance of the particle from the surface of the body (Fig. 6). Here once more we find that the most rapid flow of water occurs in the region of the tips of the comb plates, the speed decreasing nearer to the body and further away. Moving particles were visible at the extreme edge of the film, so it is not certain how far away from the body the water flow extended, although it is certainly active at a distance from the body equal to twice the length of the plates. Because of the backward movement of the water caused by the recovery strokes (the cause of the kink in the curve for the faster beat at about 1/3 of the length of the plate), it appears here that there is a greater net forward movement of water beyond the tips of the plates than within the area swept by these compound cilia. Perhaps it should be pointed out that although the distance a particle moves in one cycle of beat is similar for the two frequencies of beat shown, in one case the particles are moved this distance 5 times a second and in the other case 12 times a second.

The flow of water that is caused by the beating of ctenophore comb plates is not as smooth as that found in *Stentor*, because the distance and time interval between successive metachronal waves are much greater, but the higher the frequency of beat of the plates the more regular the flow becomes. The metachronism of beat is important not only because the time relations between immediately adjacent cilia allow a "paddle-wheel" action involving successive plates, but also because the fastest flow of the whole cycle, which is that which occurs towards the end of the effective

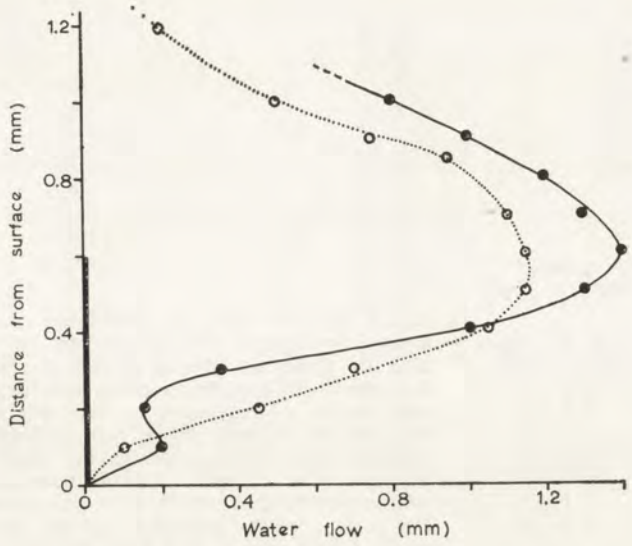

Fig. 6. The relation between the distance that particles are carried in the water flow during one cycle of beat of a comb plate of *Pleurobrachia* and the perpendicular distance of the particles from the body surface. The dotted line is drawn from information on comb plates beating at 5 cycles/s, and the continuous line on plates beating at 12 cycles/s. The length of a comb plate is indicated on the ordinate axis

stroke, can pass with little hindrance over plates that are bent low in their recovery strokes. In functional terms the plates may not be adequately considered in isolation, for many plates work together in an integrated system in which the timing of the beat of each plate is very important for the efficiency of the whole system.

Lateral cilia on the gill of *Mytilus*

These cilia are arranged in a narrow tract running along the lateral surfaces of each filament. The entire gill system comprises eight lamellae, each composed of many filaments lying side by side so that their lateral cilia face each other across an interfilamentar space. Two adjacent filaments are shown in Fig. 7. The effective stroke draws water in from the frontal surface, which faces the mantle cavity, and drives it abfrontally through the interfilamentar space which is continuous with the lumen of the excurrent siphon.

It was not possible to observe suspended particles in the stream of water passing between adjacent filaments because any particle large enough to be observed was caught and removed by the latero-frontal cirri which form a meshwork over the entrance. However, by removing a single filament and placing it on one side particles could be observed passing over the side which faced up. By focusing the microscope at a level just above the tips of the latero-frontal and lateral cilia, particles could

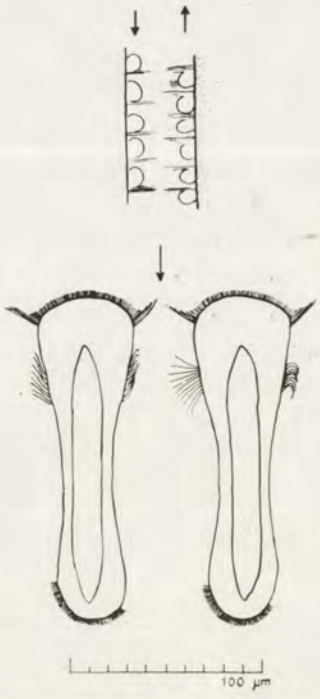

Fig. 7. *Mytilus edulis* gill. The lower part of the figure shows two adjacent filaments in cross section. The lateral cilia are shown in different phases of their cycle on the four lateral surfaces. From left to right they are: beginning the effective stroke, ending the recovery stroke, mid-way through the effective stroke, and mid-way through the recovery stroke. The arrow in the centre indicates the direction in which water is drawn through the meshwork of laterofrontal cirri into the inter-filamentar space. Above this is depicted the appearance of the metachronal wave of the lateral cilia, as seen from the front, when focusing to give an optical section mid-way through the lateral epithelium. The arrows at the top indicate the movement of the metachronal wave, which always passes down on the left and up on the right side of the inter-filamentar space, when viewed from the frontal surface

be observed to accelerate as they approached the frontal surface, to pass very rapidly over the laterals, and to slow down quickly as they passed beyond the abfrontal edge of the gill. Fig. 8 shows the relation between the velocity of various particles measured from cine films taken at 16 frames/s and the distance of these particles from the lateral epithelium. The velocity of particles passing directly over the lateral cilia was so great that they could not be identified in successive frames, but the graph indicates that the velocity of particles passing close to the tips of the laterals would be about 1.2 mm/s. Since the lateral cilia are about 15 µm long and beat through an amplitude of about 150°, each tip describes an arc of about 40 µm. At a frequency of 20 beats/s and with the effective stroke being completed in about one fifth of the time allotted to the entire cycle (Gray 1931), the tip velocity would be about 4 mm/s, while the velocity of particles moving a short distance beyond the tips is about 1.2 mm/s.

According to Dral (1967) a *Mytilus* 5 cm long has a gill area of about 17 cm². In our gills (Plate I 3 and Fig. 7) the area of the interfilamentar spaces at the level of the lateral cilia is about one third of the total gill area, but some of this is not available for water flow because it is occupied by cilia in their recovery stroke. We therefore estimate that the water must flow through about one fifth of the total gill surface, an area of 3.4 cm². At a velocity of 1.2 mm/s this constitutes a total flow of 1.5 l/h, a pumping rate in the middle of the range of values reported in the literature for mussels of this size (Dral 1967).

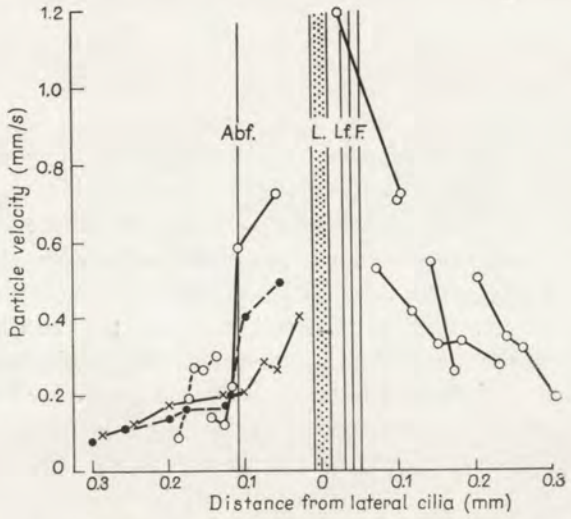

Fig. 8. A graph of the velocity of particle movement at different distances from the lateral epithelium. The graph is superimposed on a diagram of an isolated gill filament of *Mytilus*, indicating the positions of the frontal edge (F), the latero-frontal cirri (Lf), the lateral cilia (L), and the abfrontal edge (Abf). Each point represents the position and velocity of an individual particle measured from successive frames of cine film. The lines connect the same particle where such identification was possible. Circles, dots and crosses have no special significance, but are merely used to help follow the lines

With regard to the metachronism of these lateral cilia, it can be seen that this mechanism ensures the application of a steady force to the water and a well dispersed strain along the filament, for at all times about one fifth of the cilia are in their effective stroke in waves spaced at approximately 13 μm intervals along the filament. The dimensions of the interfilamentar space, the ciliary length, and the metachronal wave length are such that no part of the water between the lateral epithelia is more than about 7 μm away from an effective stroke at any time. This is well within the range of water movement caused by a rapidly beating cilium as shown by the movement of particles around the tips of *Stentor* membranelles. It might seem that ideally the effective stroke on one side of the interfilamentar space should be centered opposite a recovery stroke on the opposite side of the space, but this would require a system for constantly maintaining perfect coordination between cilia on opposite sides of the filament. As first noted by Sharpey (1835), and by many others since, the metachronal waves on the two sides of the space actually move in opposite directions, and it is our opinion that this minimizes interference of effective strokes and ensures maximum utilization of the space around the ciliary tips, regardless of slight differences in frequency or wave length along the various filaments; indeed an important result of the movement of two series of metachronal waves in opposite directions is that the frequency of beat is effectively doubled because both rows of lateral cilia act on the same body of water.

Discussion

The speed of water currents caused by ciliary activity was found to be highest in the region of the ciliary tips, where the average rate of flow measured was about one quarter of the velocity of the ciliary tip in each of the three examples. In spite of the discontinuous propulsive activity of the individual cilia, the water flow was continuous because of the metachronal timing of the beating of adjacent cilia, although the flow became less regular in the ctenophores when the frequency was low and the metachronal waves were far apart. The maintenance of a regular flow is probably the main contribution of metachronism to the functional efficiency of ciliary systems. While the speed of water movement was maximal at the level of the ciliary tips, the region of forward water movement extended well beyond the zone swept by the cilia, as far as 2–3 times the ciliary length in the two examples where particle flow at this distance could be observed. In both cases the extent of this water flow indicated that a greater volume of water was moved in unit time in the area beyond the ciliary tips than within the zone swept by the cilia, and the movement of cilia in the recovery strokes clearly contributed to the retardation of water flow close to the body surface.

In the case of *Mytilus* we have shown that the measured speeds of water flow caused by the lateral gill cilia could produce the pumping rates observed in mussels. By a similar means it is possible to calculate that the total water flow caused by the membranelles of *Stentor* amounts to about 0.1 mm^3/s, although only probably 1/3 to 1/2 of this volume is effectively filtered for food. The data given here also have implications in calculations of work done in ciliary movement (e.g. Sleigh and Holwill 1969); since the cilia are moving in a flowing stream of water in both effective and recovery strokes, the viscous work done will be less than that calculated in the effective stroke, and more than that calculated in the recovery stroke.

Acknowledgements

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Summary

An account is given of the pattern and rate of flow of water around membranelles of *Stentor*, comb plates of *Pleurobrachia* and lateral gill cilia of *Mytilus*. In all cases the water flow is most rapid in the region of the ciliary tips, where it has an average speed of about one quarter of the tip velocity of the cilium. At least half of the volume of water moved lies beyond the zone actually swept by the cilia. The water flow caused by ciliary activity is found to be more or less regular, and it is suggested that the primary function of ciliary metachronism is to convert the discontinuous activity of the individual cilia into an overall continuous movement. The flow of water caused by the lateral cilia of *Mytilus* correlates with observed pumping rates.

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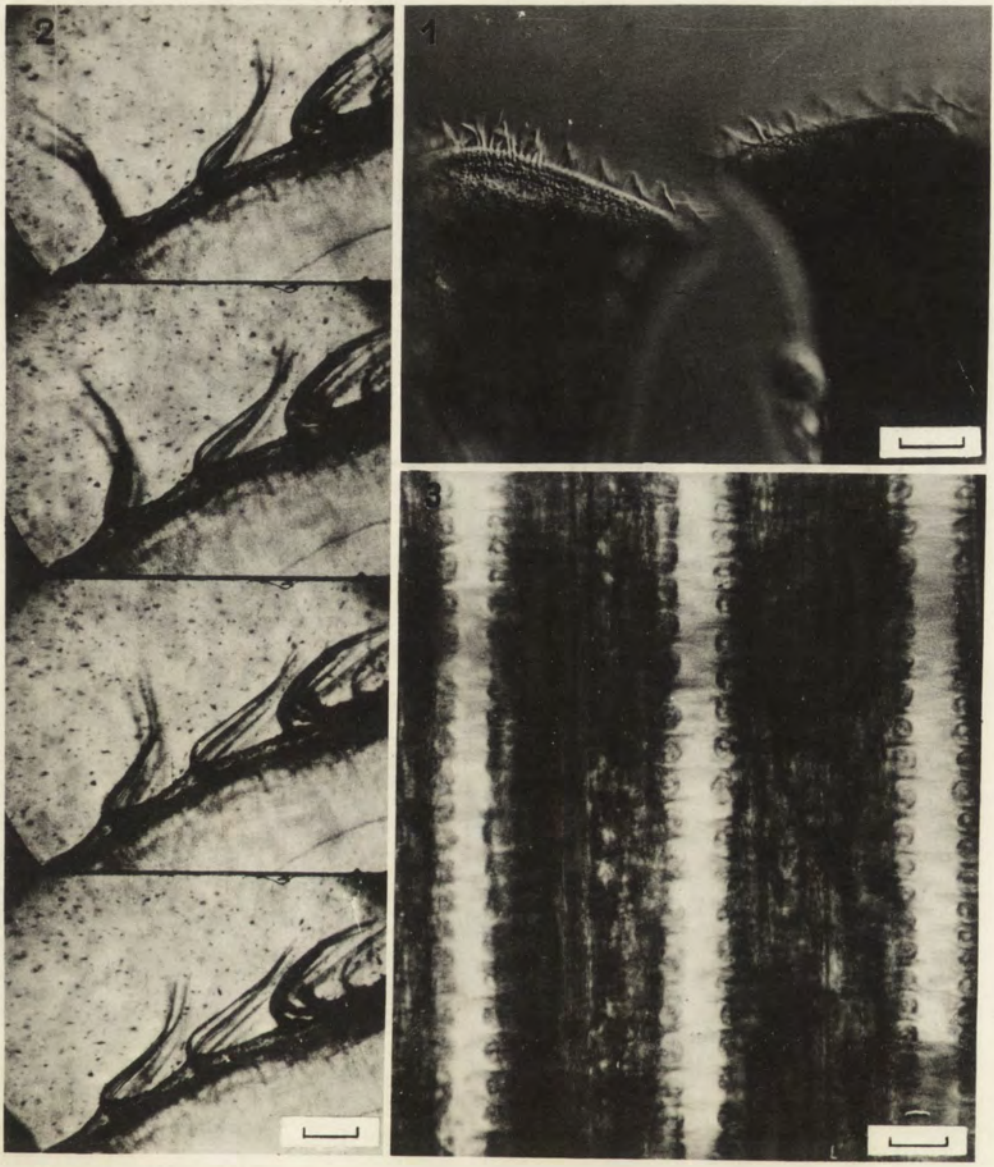
EXPLANATION OF PLATE I

1: Photomicrograph of the oral ends of two *Stentor polymorphus* showing the metachronal waves of membranelles (scale = 50 μm)
2: Photomicrograph sequence of 4 successive frames from a cine film of *Pleurobrachia* comb plates beating in water containing suspended particles; the orientation is the same as in Figs. 4 and 5 (scale = 200 μm)
3: Photomicrograph of a portion of an isolated gill of *Mytilus* viewed from the front. The lateral metachronal waves pass down on the left and up on the right of each interfilamentar space. The latero-frontal cirri are in front of the laterals, out of the plane of focus. The filaments are connected laterally by tufts of cilia (ciliary junctions) placed well below the lateral cilia and seen here as dark shadows (scale = 20 μm)



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Peter SATIR

The sliding microtubule hypothesis of ciliary motion

Basis of the model

Cilia are active organelles

The maintenance of amplitude of bend along a 50 μm or so cilium or sperm tail implies from hydrodynamic calculations that the cilia themselves are active motor organelles. Recently, Goldstein, Holwill and Sylvester (1971) have demonstrated that when the uniciliate protistan *Crithidia* is subjected to laser microbeam irradiation under conditions that sever the cilium from the cell body, the severed cilium will initiate and propagate bends and swim away by itself.

The axoneme alone is responsible for motility

During the preparation of ciliary models, the ciliary membrane is effectively broken or removed by treatments with glycerine (cf. Satir 1965 a) or triton (Gibbons et. al. 1970). The models are in effect naked axonemes that beat and swim normally when placed in ATP solutions containing the proper cations. Thus, the machinery responsible for motility resides in the axoneme alone. The ciliary membrane serves to prevent ATP from falling below a critical threshold, to provide the proper ionic environment for ciliary ATPase activity and perhaps in behavioral phenomena, such as the determination of effective stroke direction, that may depend upon membrane potential. A corollary conclusion from experiments with ciliary models is that:

Hydrolysis of ATP provides the energy for bending

The main ciliary ATPase, dynein, isolated by Gibbons (1963), comprises two rows of arms that lie along one side of each of the nine peripheral axonemal microtubules in all motile cilia. The purest preparations of dynein are capable of hydrolyzing about 13–35 ATP per second (Gibbons 1966). Since cilia beat at rates in this range, an empirical rule has been formulated that 1 ATP molecule is hydrolyzed by each dynein in an axoneme at every beat. The frequency of ciliary beat is appa-

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

rently limited by the available ATP concentration. At ordinary frequencies, the energy generated at these rates of hydrolysis appears to be sufficient to account for the work done by a cilium during a beat.

Microtubules do not change length during bending

Microtubules, composed of proteins of the class "tubulin", are the main structural elements of the axoneme, and provide attachment sites for the other axonemal components. The peripheral nine microtubules are doublets, composed of subfiber *a*, a complete microtubule and subfiber *b*, an incomplete microtubule. Sites of permanent attachment for dynein arms and radial spokes are found along subfiber *a*. The peripheral microtubules usually surround a central singlet pair in the well-known 9+2 arrangement (Fig. 1).

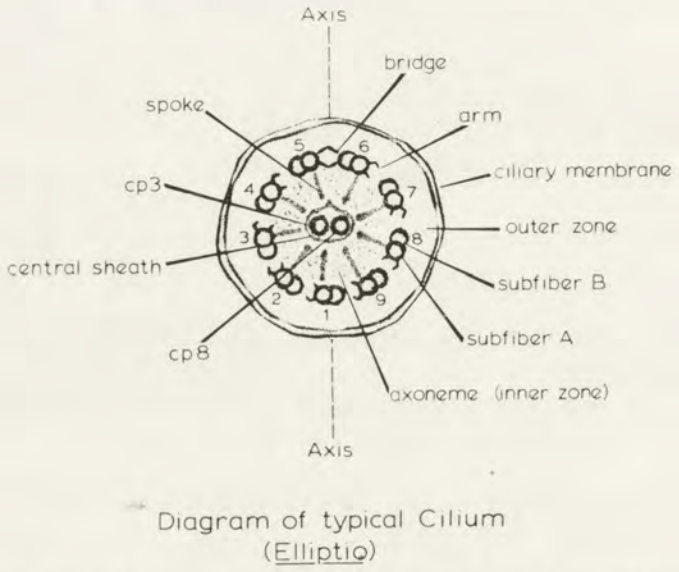

Fig. 1. Diagram of axonemal cross-section of somatic cilium viewed base to tip

The axis of the cilium bisects the ciliary cross-section in the direction of the ciliary beat. The peripheral doublets are arranged so that the axis passes between two doublets on one side of the axoneme and transects one doublet on the opposite side. The transected doublet is conventionally labeled No. 1. Numbering proceeds so that subfiber *b* of doublet No. 2 is apposed to subfiber *a* of filament No. 1. In mussel gill cilia, filament No. 1 lies to the recovery side of the ciliary stroke.

The sliding microtubule hypothesis states that the force responsible for ciliary motility is produced when the major structural elements of the axoneme, which do not change length, tend to slide with respect to one another. According to this theory, unspecified cross bridges attached to the microtubules would interact either

with a succession of sites on another microtubule or a matrix element to cause bending. Although the cross-bridges might undergo length changes or other distortions, the microtubules themselves would not appreciably shorten. This suggestion bears a formal resemblance to the sliding filament theory of muscle contraction.

For the lateral cilia of a freshwater mussel, it has proved possible to demonstrate that the doublet microtubules behave in a manner consistent with the sliding hypothesis (Satir 1965 b, 1968). The hypothesis predicts that in different stroke stages the morphological relationships of the microtubules will change in small and systematic fashion so that both qualitatively and quantitatively the geometry of the cilium will be related to the amount of sliding. One simple illustration of the qualitative relationship (Fig. 2) is that if, for example, subfiber *b* of doublets No. 1 and 6 were of equal morphopoietic length in a straight cilium, then in oder to bend the

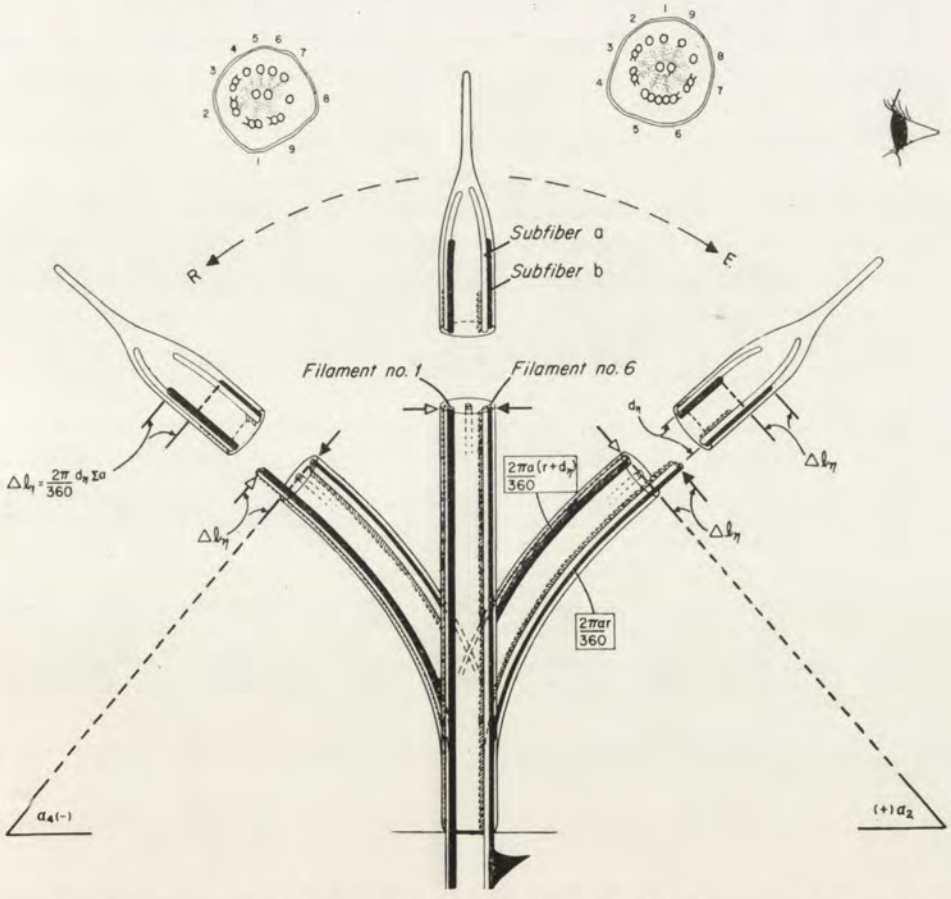

Fig. 2. Sliding filament model. Behaviour of doublets No. 1 and 6 at the tip when a cilium is bent to either side of a straight position. Bending produces an apparent length difference (Δl_n) in subfiber *b* that can be visualized in cross-sections at an appropriate level of the cilium tip. (Reprinted from J. Cell Biol.)

cilium in the direction of its effective stroke (E-pointing) subfiber *b* of doublet No. 6 would slide out past the end of subfiber *b* of doublet No. 1. To bend the cilium in the opposite direction (R-pointing) No. 1 would slide out past No. 6.

This can be visualized in appropriately fixed cilia in cross sections where some of the subfiber *b*'s have terminated. An observer standing on the effective stroke side of the cilium will see E-pointing cilia facing him, R-pointing cilia looking away. He can easily recognize which is which in a cross-section. As predicted by the theory, in E-pointing mussel gill cilia, subfiber *b* of doublet No. 6 appears longer than subfiber *b* of No. 1, while the reverse is true in R-pointing cilia (Pl. I).

For a cilium composed of straight regions and circular arcs, the total sliding of any filament relative to filament No. 1 will be given by the equation

$$\Delta l_n = d_n \Sigma\alpha, \tag{1}$$

where Δl_n is the sliding of doublet No. n in μm. $\Sigma\alpha$ is the sum in radians of the angles subtended by the circular arcs, and d_n is the distance in μm between doublet No. n and 1 projected on the axis of the cilium. For doublets 6 vs. 1, d_n is approximately the axonemal diameter (0.2 μm). In E-pointing cilia with a single bend α may be maximally about 100°, which is arbitrarily designated +100°. In R-pointing cilia, α may be minimally −80°. Therefore Δl_n varies from about +0.35 to −0.28 μm. A negative Δl_n means that doublet No. 1 is sliding. Where this has been examined in detail, the quantitative correspondence between predicted and experimentally-determined values for Δl_n is very good (Fig. 3). The doublets move as integral units.

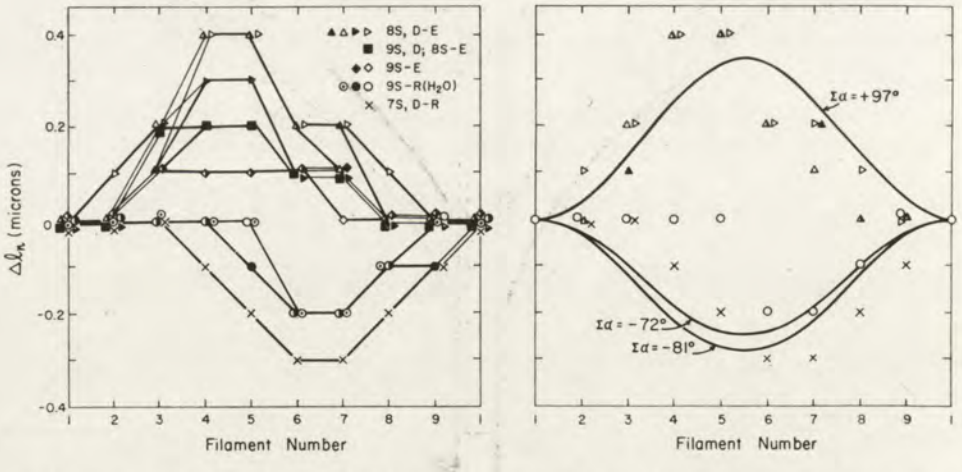

Fig. 3. Comparison of prediction of sliding model to reconstructed ciliary tips. Complete method and notation given by Satir (1968). Left—measured points in E vs. R cilia. Right—for maximum bending in E and R direction, predicted values (curves) match measured points. (Reprinted from J. Cell Biol.)

Features of the model

Sliding is caused by an active process that generates longitudinal shear throughout the axoneme. Bends are produced by the presence of longitudinal shear resistance (Brokaw 1971). In most cases, the axoneme is structurally unique at the juncture of cilium and cell. This may reflect increased shear resistance and explain why bending usually begins at the base of the cilium.

We do not yet know what interaction causes active sliding. The possibilities that have been suggested include:

(a) microtubule-matrix interactions, that would most probably involve sliding of the radial spokes along the central sheath;

(b) microtubule-microtubule interactions, that involve dynein arms from subfiber *a* of one doublet sliding along subfiber *b* of the adjacent doublet;

(c) microtubule-microtubule interactions, that involve dynein arms from one doublet sliding along the radial spokes of the adjacent doublet.

Since dynein is the major, if not the only, axonemal ATPase, it seems likely that the arms must be part of the sliding interaction. Where dynein has been removed from the axoneme, the axoneme straightens. No reports have appeared on the activation of dyneinless models as yet, and this might provide a critical test of the importance of dynein. One difficulty with possibility (b) has been that dynein arms are rarely seen to bridge the space between adjacent doublets, even in bent regions of the axoneme. The inner dynein arms are also only occasionally attached to the adjacent spoke. However, predictions of the number of dynein arms that would be attached at any one time are low.

ATP is presumably dephosphorylated at every attached interaction site along each doublet every time the doublet slides by one arm period ($\sim$175 Å). Each model of interaction gives certain predictions of the maximum amount of ATP that would be utilized. We can arrive at a rough estimate of the maximum total microtubule sliding during a single beat in the mussel gill cilium by adding the absolute values of Δl_n for every doublet in cilia maximally bent in E-pointing and R-pointing directions. The sum is about 2.7 μm or about 0.3 μm per doublet, which represents the maximum spacing of attached interaction sites. This spacing is reduced by a factor of 2–3 in some models. Some structurally possible sites of attachment can be empty during any interaction cycle should there be a vernier alignment of arms and interaction sites. Either the spokes or sites along subfiber *b* can be approximately fitted to this information.

The ciliary wave form could be produced either by (1) microtubules on differing sides of the cilium actively sliding in the same direction or (2) microtubules on one side actively sliding in different directions at different beat stages. Since the polarity of subfiber *a* is probably reflected mechanistically, it seems likely that active sliding is in one direction only. Doublet microtubules on opposite sides of the axoneme

sliding in one direction at different times during a single beat would produce bends in opposite directions.

The control system that activates microtubule sliding remains obscure. Most likely, the primary controls of activation lie within the axoneme proper and mechanical feedback accounts for the sequence of activity. This is consistent with the idea of intrinsic mechanosensitivity of the axoneme. Superimposed on the mechanical features are cellular controls of ATP and divalent cation concentration that turn the cilium on and off or change effective stroke direction.

The sliding microtubule model accounts for the constancy of axonemal diameter in 9+2 cilia since d_n is a parameter of the fundamental equation of sliding. The model supports the hypothesis of a constant basic mechanism of motility for all cilia, although specific morphological or environmental differences may alter the precise appearance of the beat from one type of cilium to the next. The model predicts certain relationships between axoneme bend and tip or basal body configuration in all cilia. These relationships seem to hold generally.

The model apparently also accounts for the active bending moments produced during motion of invertebrate sperm tails (Brokaw 1971, Rikmenspoel 1971). In the absence of silding, the model predicts that a constant angle will be maintained between two ends of a cilium as waves traverse it. In thiourea-treated sperm tails or in most sperm tails that propagate bends after laser microsurgery, this seems to be the case. A feature of the model is adirectionality in the sense that neither the direction of sliding nor the direction of bend propagation is specified.

During sliding, permanent connections between microtubules would become stretched. If, for example, the radial spokes were permanently attached to the central sheath, as sliding proceeded the spokes distal to a developing bend would be extended. Consider an idealized cilium of diameter d and spoke length $d/2$ in a straight region where the spokes are perpendicular to the microtubules (Fig. 4). If the spokes remain permanently attached to the central sheath, distal to a bend of angle α they would be of length s, where

$$s^2=(d/2)^2+(\Delta l_n/2)^2. \tag{2}$$

In addition, the spokes would no longer be perpendicular to the microtubules, but instead would make angle θ with subfiber a. In the direction of bend, where $d/2 \sim d_n/2$

$$\cot\theta=\alpha(\alpha \text{ in radians}). \tag{3}$$

In straight regions of mussel gill cilia, both proximal and distal to a single bend on the cilium, the spokes are perpendicular to the microtubules. Therefore, as sliding proceeds in straight regions, the spokes are not continuously attached to the central sheath. In bent regions, however, the spokes do not remain perpendicular to the microtubules. Preliminary evidence suggests that in these regions θ changes as predicted by equation 3, and that at least some of these spokes do remain attached

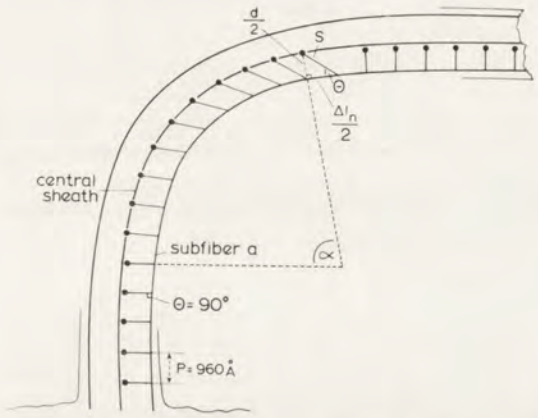

Fig. 4. Schematic model of spoke attachment in an idealized cilium with a single bend. Major spoke period (p) is indicated

as sliding proceeds. Fig. 4 shows a model of spoke attachment as it is presently conceived. This suggests that the spokes play a role in maintaining the form of the bend and in bend propagation. It is also consistent with the non-uniform development of a bend with continued sliding since, as Δl_n increases linearly, the spokes are stretched according to a formula that is non-linear. In this regard it is interesting to note that the minimum radius of curvature occurs when Δl_n is about twice the spoke period, and spoke stretch is no greater than 40%.

Satir and Gilula (1970) have described the presence of specific connections between microtubules and the membrane at the distal edge of the basal body of many somatic cilia that correspond to an array of particles seen within the membrane termed the ciliary necklace (see B. Satir, this symposium). If these attachments between membrane and microtubules were permanent, as appears to be the case in the necklace region of somatic cilia, sliding of the microtubules would necessarily stretch these connections in a manner similar to the stretch of the radial spokes. This might affect the alignment of the membrane particles within the necklace and could be a mediator of ciliary stroke. Mechanical distortions of the axoneme near the tip would, according to the model, initiate sliding along the entire axoneme and thus change the necklace relationships. Where bending in specific directions was coupled to membrane depolarization, the cilium would be an effective directional mechanoreceptor. The model therefore provides some rationale for energy transduction in cilia-based sensory systems such as the insect campaniform sensillum or the hair cell of the vertebrate ear.

Acknowledgements

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Summary

Cilia are active organelles whose 9 + 2 axoneme is alone responsible for motility. ATP hydrolysis by ciliary ATPase (dynein) provides energy for bending. Axonemal microtubules do not change length as the cilium moves from one position to another. This suggests that the force responsible for ciliary motility is produced when the microtubules tend to slide with respect to one another. This hypothesis accounts for features of ciliary motility including constancy of axonemal diameter and bending properties of invertebrate sperm tails, and also provides a rationale for energy-transduction in certain cilia-based mechanoreceptors.

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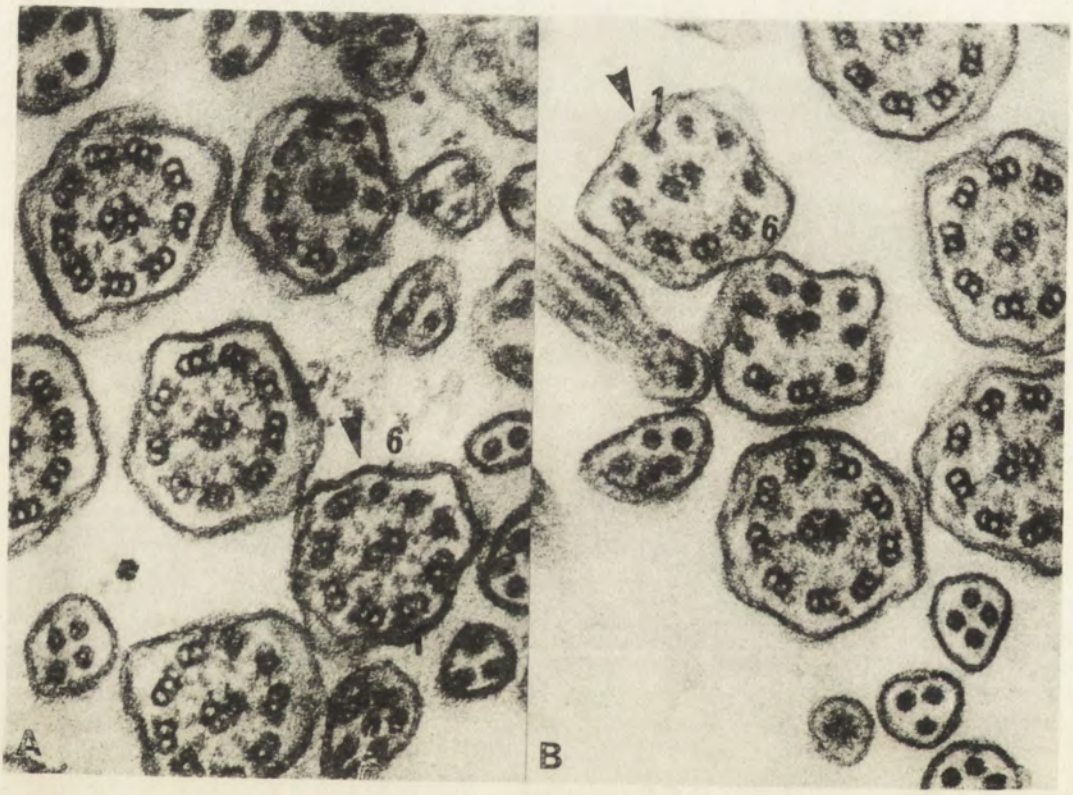
EXPLANATION OF PLATE I

A—R-pointing lateral cilia. B—E-pointing lateral cilia. Arrows indicate cross-sections corresponding to Fig. 2. × 95 000



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Laser induced neuroid transmission in a ciliated epithelium

Neuroid transmission may be defined as conduction between epithelial cells analagous and possibly homologous to conduction through nerve and muscle. The subject has recently been reviewed by Mackie (1970). In 1932, A. M. Lucas described a spontaneous arrest of *Modiolus* gill cilia that propagated across cell boundaries, which he believed to be evidence for neuroidal control of ciliary beat. A similar phenomenon was described by Nelson (1951) in oyster gills. We have found that this arrest can be produced at will following laser microsurgery under defined ionic conditions in the gill filaments of the freshwater mussel (*Elliptio* sp. or related genera).

Method

Small pieces of gill are prepared as described by Satir (1963). Aliquots of tissue are activated in solutions containing cations and drugs appropriate to each experiment and placed in a non-toxic blue dye (Brilliant Blue F. C. F.) on depression slides. The slides are transferred to the stage of a Zeiss phase contrast microscope upon which is mounted a biolaser (Control Data) that emits a short pulse of 694 nm red laser light. Laser emission is directed down the microscope by a mirror and focussed by the optics of the microscope to a small spot. The experimental setup derives from the work of Goldstein (1969) on laser microsurgery of sea urchin spermatozoa. The radiation is absorbed by the dye and will produce local lesions in the gill epithelium. Regions of filaments whose lateral cilia are beating with good metachronal rhythm are chosen for irradiation. The beam is focussed at the surface of the lateral cells near the center of the chosen region.

Results

When the activating solution contains 15–20 mM K^+, usually as KCl, the irradiation permanently destroys a local portion of the gill filament, about 30 μm long under our usual experimental conditions. The damaged region often bulges out and

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

cells are expelled from the epithelium. The morphology of cilia in the lesion has been examined (Goldstein and Satir 1971). The metachronal wave continues to either side of the damaged region (Text fig. 1); occasionally we observe an increase in wavelength downstream from the lesion.

When the activating solution contains 15–20 mM Na^+ – as NaCl, $NaHCO_3$, or Na_2SO_4 – or 5–15 mM Ca^{++}, as $CaCl_2$, the irradiation not only produces

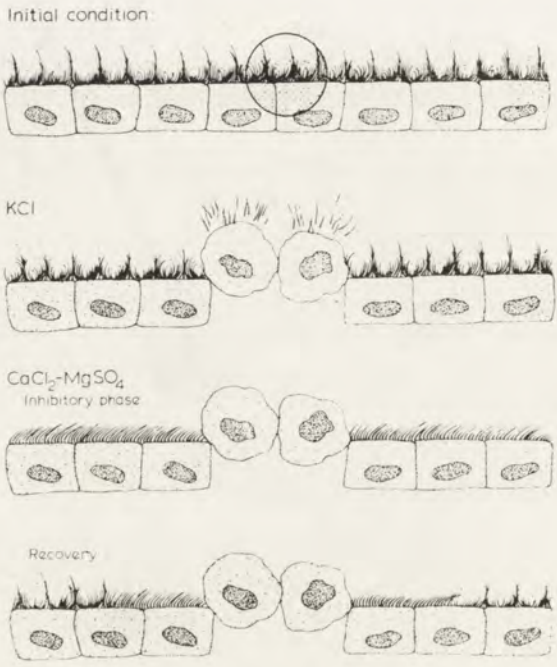

Fig. 1. Diagram of laser experiments. Uppermost figure indicates condition of the gill filament prior to laser irradiation. The circle indicates the size of the lesion. Lower diagrams show typical results in 15 mM KCl and 8.75 mM $CaCl_2$–1.75 mM $MgSO_4$ solutions respectively. Upon completion of recovery, the tissue in $CaCl_2$–$MgSO_4$ is indistinguishable from that in KCl

local damage, but it also causes a wave of inhibition of ciliary activity to spread along the gill filament. The rate of spread can be measured from high speed films (Pl. I 2) to be from about 100–500 μm/sec. Sometimes, spreading is so slow it can be followed by eye. The inhibition moves out for distances of greater than 1 mm to either side of the lesion. Since the average cell length is about 25 μm, at least 50 cells in a single row are systematically affected. The lateral cells are four rows deep; usually all four rows are inhibited. The inhibition will not pass a physical break in the epithelium.

The cilia appear to be stopped in a single stroke position. The arrest lasts for a few seconds (usually about 10–15) and then recovery begins from the edges of the

inhibited region. This suggests that the underlying basis for the arrest is a cellular phenomenon that is decremental with distance. Within a minute after the hit, the cilia all beat normally again, except in the damaged region (Pl. I 3). The experiments are usually run in solutions containing 1 mM serotonin (5-hydroxytryptamine) and 1 mM tris buffer at pH 7.5. Neither of these components has any pronounced effect on the phenomenon.

The arrest does not occur in solutions containing NH_4^+ or Mg^{++} as the principal cations, which give results identical to K^+. In preliminary experiments other divalent cations, such as Zn^{++}, when not toxic, permit arrest. Solutions containing 1.75 mM $MgSO_4$ and 8.75 mM $CaCl_2$ do not differ from solutions containing Ca^{++} alone in their ability to permit arrest.

The gill is innervated, but nerve poisons tested so far in solutions containing Na^+ or Ca^{++}, including 10^{-6} M tetrodotoxin, 10^{-5} M ouabain, and 10^{-4} M eserine, have no effect on the spread of inhibition.

Discussion

The laser-induced ciliary arrest appears to be a true neuroidal phenomenon in that the underlying cellular event probably spreads directly from epithelial cell to epithelial cell. In life, the phenomenon may be triggered by nerve impulses, since the epithelium is innervated and the arrest is known to occur spontaneously. The cells of the gill epithelium are electrically coupled and possess at their borders specialized cell junctions usually considered responsible for coupling (Gilula and Satir 1971). The epithelium probably provides a continuous channel for the movement of ions, uninterrupted by cell boundaries.

It first appears that the arrest could be accounted for if the laser opens up the cytoplasm of a few cells momentarily to external ions which then diffuse away from this spot along the epithelium. This is unlikely to be the case since diffusion over millimeter distances should be much slower than the observed rate of spread.

Another possibility is electrotonic spread. Mechanical distortion of the anterior end of the cell membrane of *Paramecium* has been shown by Eckert and Naitoh (1970) to cause momentary ciliary inhibition throughout the cell. This is due to a rapid increase in Ca^{++} permeability that results in membrane depolarization. The depolarization spreads throughout the cell at high speed with little ($<5\%$) electrical loss so that measurements at electrodes placed 100 μm apart are only about 1 msec out of phase. Since this rate is much faster than the metachronal wave velocity, Eckert and Naitoh rule out a neuroidal basis for metachronism in *Paramecium*.

In the gill epithelium, the spread of ciliary arrest is much slower than would normally be associated with electrotonic spread, and instead approaches metachronal wave velocity. It may be that the experiments reported here are indicative of a hitherto unexplored cellular transmission mechanism common to ciliated cells.

The records obtained by Satir and Miller (cf. Satir 1965) could be associated with this phenomenon.

It is clear from the geometry that the turning on and off of the gill cilia is a finely controlled process, particularly since four rows of cells must be turned off in unison. The metachronal wavelength of these cilia is shorter than the length of a single lateral cell. It would be interesting to know whether portions of a single cell can be arrested at different times, but we have been unable to determine this at our present level of resolution.

Acknowledgements

We thank Michael Gurfinkiel for assistance. This work was supported by USPHS grant HL13849. The laser was purchased with funds provided by USPHS FR 7006.

Summary

When mussel (*Elliptio*) gill lateral cells are activated in 15–20 mM K^+ (KCl) or NH_4^+ or Mg^{++}, laser microbeam irradiation produces a small (30 μm) local lesion, to either side of which the metachronal wave continues. Irradiation of gill activated in 15–20 mM Na^+ (NaCl, $NaHCO_3$, Na_2SO_4) or 5–15 mM Ca^{++} ($CaCl_2$) produces not only local damage, but also a wave of ciliary inhibition that spreads out at rates of 100–500 μm/sec for distances of >1 mm to either side of the lesion. The arrest lasts for 10–15 sec; recovery begins from the edges of the inhibited region. The underlying event causing arrest propagates across cell boundaries probably directly from one epithelial cell to its neighbors.

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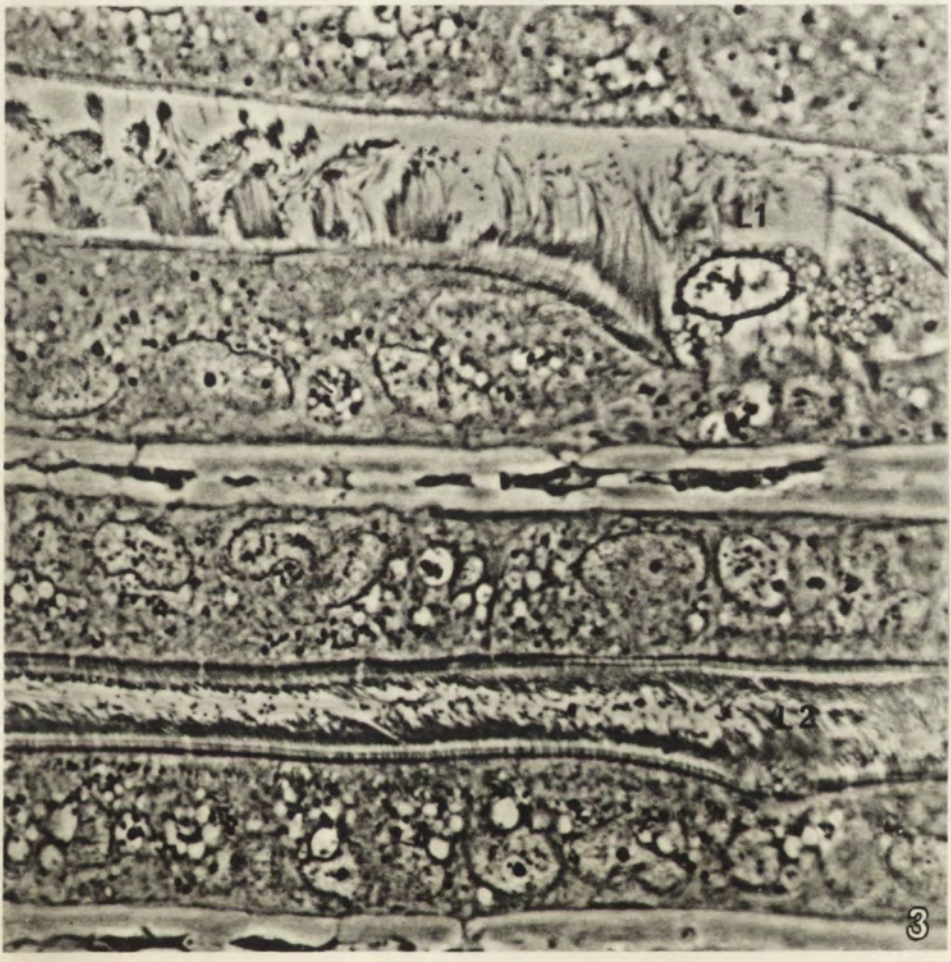
EXPLANATION OF PLATE I

2: Sequence of frames from film of arrest in $CaCl_2 - MgSO_4$ solution. Arrow indicates center of laser hit. Times—24,64,144 msec after hit. Calibration—metachronal wavelength = 10 μm

3: Light micrograph of $CaCl_2 - MgSO_4$ experiments. Material has been fixed, embedded and sectioned. L1 marks a lesion where the tissue has been allowed to recover. Note the metachronal wave (Satir 1963). L2 marks the second lesion where the cilia are still inhibited. 1120×



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The ciliary necklace in *Tetrahymena*

The similarity and stability of the fine structure of cilia from a variety of tissues has been known and studied by transmission electron microscopy of thin sections for several decades. With the emergence of an alternate technique: the freeze-fracture, freeze-etch technique – new aspects of ciliary structure can be described. Freeze-fracture is known to split membranes to reveal internal faces (Branton 1966), while etching reveals true surfaces (Da Silva and Branton 1970, Tillack and Marchesi 1970). Specializations exist within the ciliary membrane and have been found to vary in different organisms, when present. One specific ciliary membrane specialization was first seen in clam gill cilia by Satir P. and Gilula (1970) and they coined the term the ciliary necklace for its description. In the gill, the necklace consists of three well-defined scalloped rows of 100 Å particles that encircle the cilium. The rows are located in the transition region just below the basal plate.

This paper shall deal with freeze-etch replicas of cilia from the protozoan *Tetrahymena pyriformis*. We shall focus on two aspects of the ciliary necklace: (1) its structural specificity and (2) some early steps in its formation.

Method

Cultures of *Tetrahymena* grown axenically on 2% proteose peptone were fixed in 0.5% glutaraldehyde in 6–50 mM phosphate buffer pH 7 for half an hour at room temperature, then transferred to 20% buffered glycerine for 2–3 h or overnight. The cells were then frozen in Freon at −150°C, and stored at liquid nitrogen temperature. The frozen specimens were fractured in a Balzers apparatus at −115°C, with or without slight etching (ca. 15 sec).

Results

The freeze-fracture appearance of cilia from *Tetrahymena pyriformis* is shown in Pl. I. The majority of longitudinal fractures pass through the ciliary membrane which usually appears smooth with only a few particles associated with most of its length. Near the juncture of ciliary and cell membrane, the ciliary necklace is present in

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Tetrahymena. Pl. I shows the two strands characteristic of the necklace in *Tetrahymena* on the particle-rich fracture face (A) which faces the axoneme. The strands are 250 Å apart and they are composed of packed ca. 100 Å diameter particles. They are not scalloped. The necklace is present on all oral and somatic cilia examined. The membrane surface (S) revealed by etching can also be seen. In etched preparations, microtubules of the basal bodies are often preserved especially well and a ca. 50 Å microtubular subunit can be visualized (Pl. II).

Satir P. and Gilula (1970) have shown that the necklace corresponds to a unique cross-sectional appearance of the cilium. Pl. III shows a longitudinal cut of *Tetrahymena* displaying several kineties in cross-section at different levels. The familiar 9+2 pattern can be seen in the top row, and (at arrow) the definitive cross-section of the necklace region is seen. The section lies between the basal plate – that is, the end of the central pair – and the beginning of the basal body triplets. In this section, the nine doublet microtubules retain their basal body positions and a series of specialized connections runs from the midwall of each doublet to the membrane.

Pl. IV shows a freeze-fracture replica of alveolar membranes and of the B face of the cell membrane, which faces the exterior. The cell membrane is fractured along a primary meridian, and two cilia (Ci) are cross-fractured. The parasomal sac at one edge of each cilium is apparent. In between the cross-fractured cilia is a region of the membrane where we hypothesize that a new cilium will grow. The parasomal sac is already evident and careful observation reveals a B face appearance of the ciliary necklace. There are fewer particles associated with the B face, but the complete circumference of the necklace can be seen.

Discussion

The ciliary necklace has been described as a standard feature of somatic cilia but it is absent in sperm (Satir P. and Gilula 1970, Gilula and Satir P. 1972). In contrast to the necklace in the mussel gill, in *Tetrahymena* the necklace is comprised of only 2 strands and scalloping is not apparent. The same number of strands is present on both oral and somatic cilia in *Tetrahymena*. The standard cross-section corresponding to the necklace is seen in *Tetrahymena* and connections between microtubules and membranes are found. Satir P. and Gilula describe these connections as shaped like a "champagne glass, the stem of which joins the microtubules, the bowl apposes the membrane". They have also been able to observe that the number of rows of connections in longitudinal sections corresponds to the number of necklace strands. We have been unable to resolve the connections in longitudinal sections of *Tetrahymena* so far.

Our pictures support the interpretation that, during fracture, the necklace region cleaves along a unique plane to give two complementary fracture faces. The particles are an internal feature of the membrane in the necklace region. Most of the necklace particles adhere to face A. However, some particles are present on face B.

Tetrahymena cilia are added asynchronously along the primary meridians as the cell grows in preparation for fission (Randall and Disbrey 1965). Fractures that reveal regions where new cilia are about to grow show rings that seem to be the forming ciliary necklace. The parasomal sac is an important feature in the identification of these newly-emerging cilia. Preliminary fractures of deciliated cells that are regenerating cilia confirm this finding. We conclude that the ciliary necklace is formed early in ciliary morphopoiesis. This would mean that the growth of the ciliary membrane either occurs above the necklace region or that small micelles incorporated at the ciliary base may move through the region without displacing the necklace particles.

Acknowledgement

We thank Michael Gurfinkiel for assistance. This work was supported by a grant from USPHS (HL 13849).

Summary

The presence of the ciliary necklace is shown in freeze-fracture replicas of the protozoan *Tetrahymena pyriformis*. The necklace is found on both somatic and oral cilia. It is composed of two strands ca. 250 Å apart. On fracture face A each strand consists of a row of ca. 100 Å tightly packed particles. In regions where cilia are about to grow, rings appear that correspond to the forming necklace. This infers that the necklace is formed early in ciliary morphopoiesis.

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EXPLANATIONS OF PLATES I–IV

1: Freeze-fracture of longitudinally cut *Tetrahymena* cilia. The ciliary necklace (arrow) consisting of two strands can be seen at the base of the cilia. The outer surface of the cilia can also be observed (S). Encircled arrowhead shows shadow direction. Magnification 72 000 ×
2: A freeze-etch replica showing the preserved basal body microtubules. At arrow the microtubular subunits can be seen. Encircled arrowhead shows shadow direction. Magnification 75 000 ×
3: Surface view of a longitudinaly cut section of *Tetrahymena* showing several kineties. Note the cross-section corresponding to the ciliary necklace region (arrow). Magnification 22 500 ×
4: Freeze-fracture of cilia (Ci) in cross-section. The fracture cleaves the cell membrane between two alveolar membranes (A). The cell membrane is intact over the newly emerging cilium. Note that the parasomal sac (ps) and the ciliary necklace have already formed. Encircled arrowhead shows shadow direction. Magnification 72 000 ×

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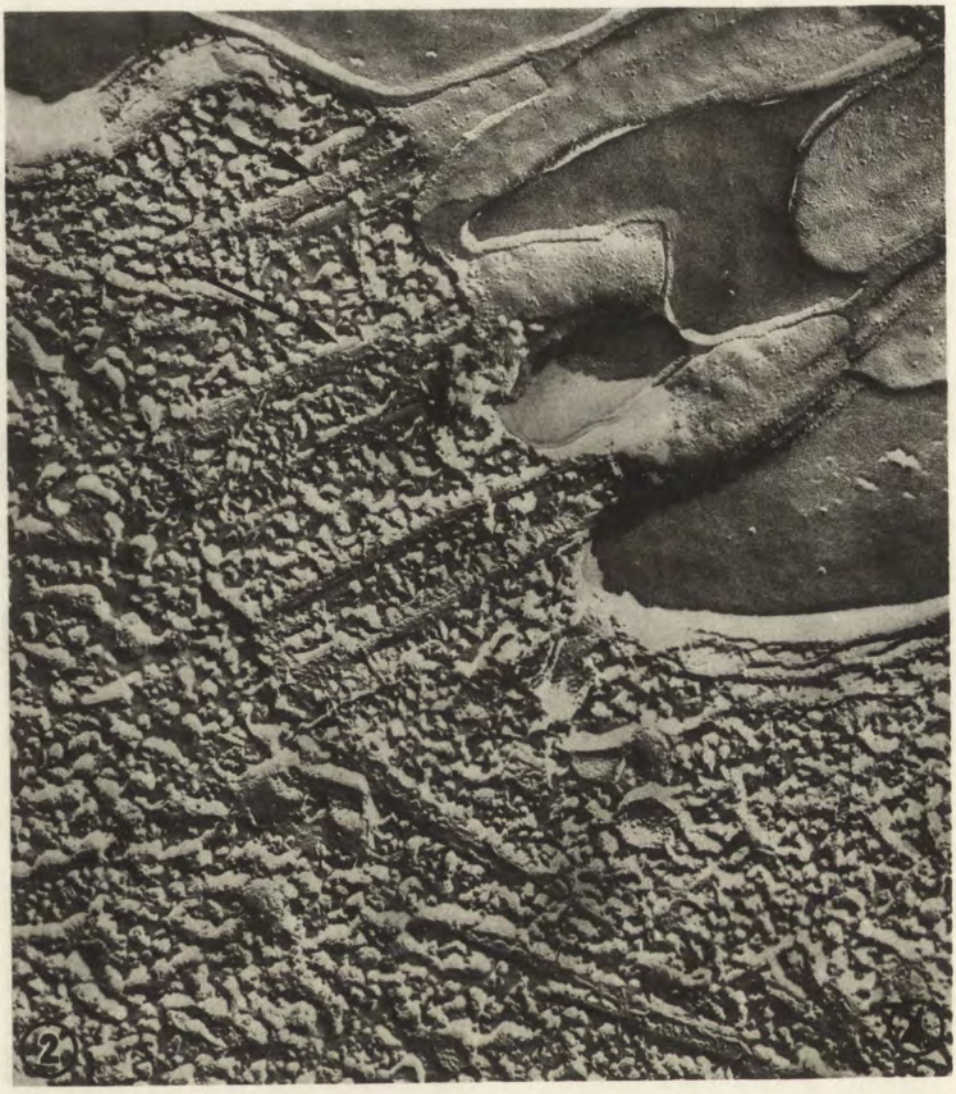

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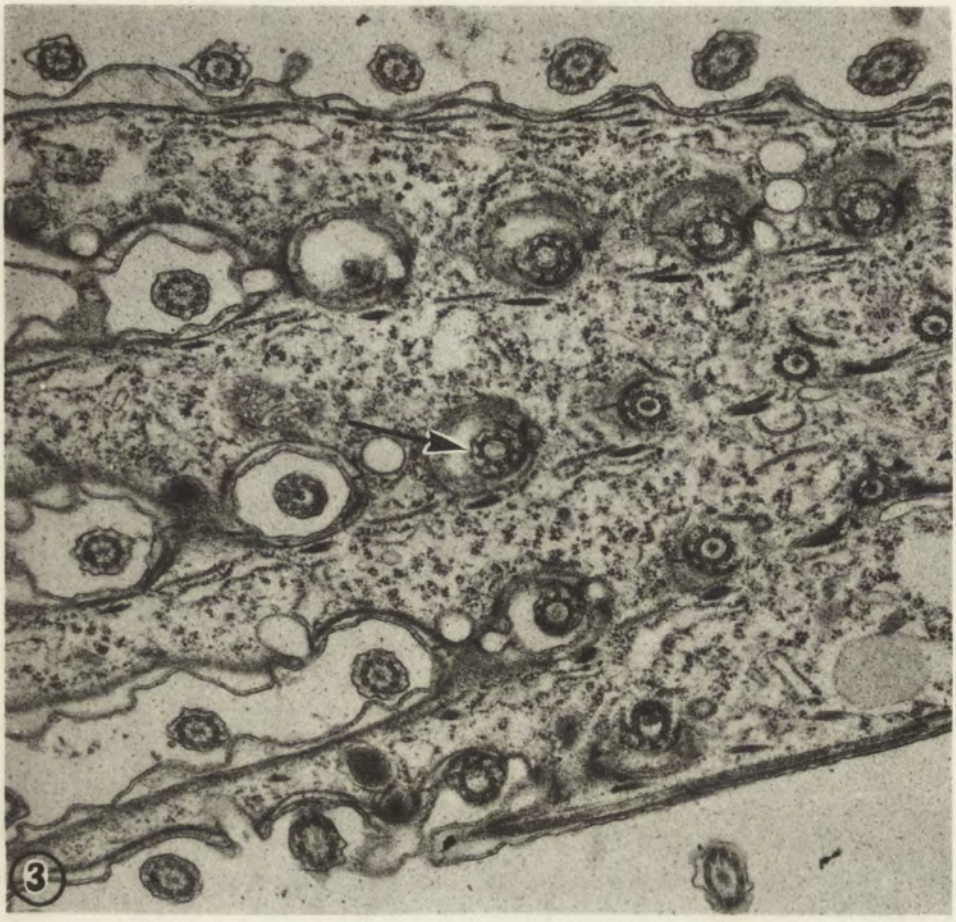

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Properties of polarized ciliary beat in *Paramecium*[2]

Improved techniques of visualizing ciliary activity in ciliates recently revived discussion of the properties of ciliary motion in paramecia (Allen 1969, Kuźnicki, Jahn and Fonseca 1969, 1970, Kuźnicki 1970, Machemer 1969, 1970). It is generally agreed, that a major difficulty in the interpretation of working cilia is the restriction to one plane of observation, due to the limited depth of focus of high power optics. How can reliable conclusions be drawn from 2-dimensional aspects of a highly complex 3-dimensional movement of cilia? Are the profiles of metachronal waves appearing at the body edges of swimming[3] and stationary cells optical illusions, or do these waves represent the true 3-dimensional patterns of polarized ciliary beating, as initially found by Párducz (1954)? And if the patterns exist, how can we determine their spatial properties from 2-dimensional photographs?

Answers to these questions, which are basic for all studies on the properties of metachronal coordination in ciliated cells, can be facilitated by using ciliary models for the purpose of comparative work. If it is possible to construct a well-proportioned model of ciliary metachrony according to instantaneous pictures of actual metachronism, then the major 2-dimensional aspect of the model should agree with corresponding profile and surface views of working cilia.

Flash-photographs of stationary and swimming paramecia under controlled experimental conditions (9 mM $CaCl_2$, 3 mM KCl, 5 mM Tris-HCl pH 7.2, 20°C), taken with Nomarski-optics on high-resolution 35 mm-film (Agfa Agepe ff) were used to build up a wire model of beating cilia. Plate I 1–3 gives full length views of 3 paramecia: (1) shows an animal during free forward swimming; *Paramecium* (2) is moving forward too, but the cell is slightly compressed; (3) represents a stationary specimen under slight compression. Profiles of metachronal waves are seen in each of the examples, but there are some specific variations in the apparent wave length and the apparent metachronal configuration.

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[1] On leave of absence from the Zoological Institute, University of Tübingen, Federal Republic of Germany.

[2] Partly supported by the Deutsche Forschungsgemeinschaft

[3] I first saw excellent profile views of metachronal waves in swimming paramecia at normal viscosity in unpublished micrographs by S. L. Tamm.

Surface-view photographs of working ciliary populations were important for the construction of the model. Plate II 4 gives a high-power surface view of the anterior end in a forward swimming specimen. The lines of ciliary synchronization and the positions of cilia during the recovery stroke (sickle-shapes) and during the effective stroke (points and rather stretched lines) can be identified in the photograph. This and other surface views correspond to well-known fixed and stained wave patterns of forward swimming paramecia (Pl. II 5).

In an instantaneous picture of a metachronal wave seven stages of cyclic polarized activity of cilia are usually seen. Seven distinct stages of model cilia were therefore shaped in a process of repeated comparison and adaptation to photographic views of working cilia. Plate III 6 gives a surface view of these model cilia. Stages 1–3 represent cilia during the effective stroke, stages 3–7 show the recovery stroke. The model cilia are arranged in their normal metachronal sequence. The effective stroke is directed at approximately 90° toward the right with respect to the direction of metachronal transmission; hence the model shows a dexioplectic metachrony. If a population of model cilia is grouped according to the pattern of Pl III 6, we get a familiar picture of ciliary metachronism in *Paramecium*. The model wave system, shown in Pl. III 7, represents a small body surface area of a forward swimming *Paramecium* at one moment.

The first step in verification of the model was to cut the wave system into a series of "optical slices" under different wave angles. The line of reference was the ciliary synchronization parallel to the wave crests. A catalogue of metachronal wave profiles results which undergo modifications with increasing wave angles (Pl. IV 8). The true wave length appears at approximately right angles to the line of ciliary synchronization. Angles near 0° and 180° bring forth very long waves, each with a characteristic grouping of cilia. The relations between apparent beat direction and apparent wave direction change with the angle of observation: At angles between 0° and 90° the metachrony appears to be antiplectic, as the effective stroke and metachrony seem to be opposed. I call these aspects of patterns "antiplectoid". Between 90° and 180°, on the other hand, the effective stroke points into the direction of metachronal transmission which is an apparent symplectic or "symplectoid" pattern. The relations between dexioplectic metachrony of body cilia and its different aspects are explained in Fig. 1.

Do some of the model views fit the observed profiles of metachronal waves? They in fact do so systematically. Pl. VI 9 gives the enlarged edge view, topographically the right body side, of *Paramecium* in Pl. I 1. Above the photograph, model views of a comparable ciliary configuration indicate that the real wave front passed forward at decreasing angle ranges from 40–60° to 20–40°. The pattern is antiplectoid. In another typical example with somewhat longer wave profiles (Pl. VI 10) it is shown, that the wave angle also decreases in anteriad direction from 20–40° to 0–20°; again there is an antiplectoid pattern. An example of symplectoid configuration from the anterior right portion of the specimen in Pl. I 2 is given in

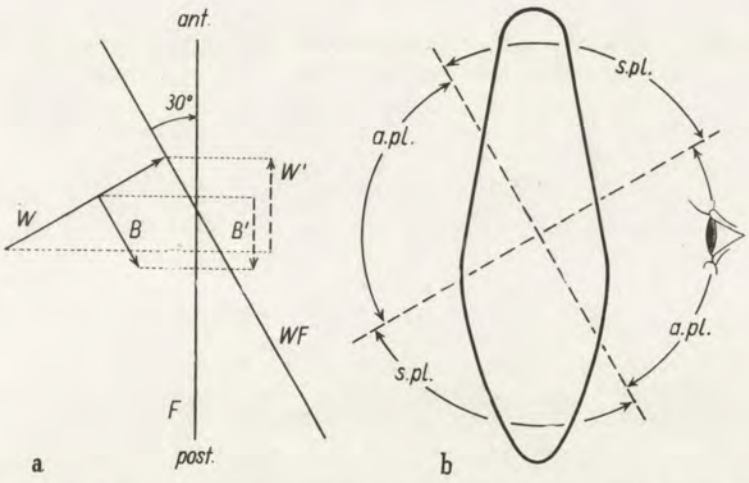

Fig. 1. (a) Relation between dexioplectic metachronism of a forward swimming *Paramecium* (solid arrows) and its projection to profile view parallel to the longitudinal body axis (dashed arrows). (b) Sectors of antiplectoid and symplectoid appearance of dexioplectic metachrony for wave fronts deviating 30° from the longitudinal axis of *Paramecium*. In the typical plane o microscopical observation antiplectoid patterns prevail. W′, projection of wave direction (W) B′, projection of beat direction (B); WF, wave front or line of ciliary synchrony; F, plane of focus of ciliary profile views; a.pl, antiplectoid sector, s.pl, symplectoid sector of views

Pl. VI 11. The corresponding model view explains that in this case the wave crest unusually passed the body edge at an angle of 160–180°.

Profile outlines of metachronal waves passing the concave ventral surface (oral groove) clearly differ from those of other regions. After completion of the effective stroke the cilia of the oral groove (which occur in pairs) straighten their shafts from base to tip more vigorously, and the angle of gyration seems to be reduced. The beat direction does not always coincide with that of the body cilia: it may be more posteriorly oriented (Pl. VI 12), or it may even point toward the anterior though the body cilia beat backward (Pl. VI 13). The modified model views (Pl. IV 8b) take account of the different recovery phase of the peristomal cilia, and allow the determination of actual wave angles, which usually are found to be in a range of 20–40° (Pl. VI 14).

Table 1 gives a quantitative evaluation of ciliary profiles of forward swimming paramecia at normal conditions. According to averaged values of 5 body sections the wave angles increased from anterior to posterior. The true wave length λ can be calculated from the apparent wave length λ' in accordance with the equation

$$\lambda = \lambda' \times \sin \alpha .$$

λ' can be read directly from profile photographs of metachronal waves; the wave angle α is derived from the model waves. The Table shows that in spite of different readings of apparent wave length and wave angle the resulting calculated body wave

Table 1

Average range of angles and length of metachronal waves of forward swimming paramecia at normal conditions (20°, 1cP), calculated from ciliary profiles and corresponding models

Body region	range of angle (α)			wave length (μm)				
				apparent		calculated	direct	
	n		$\sin \bar{\alpha}$	n	λ'	$\lambda' \times \sin \bar{\alpha}$	n	λ
ant.	10	4–24°	0.24	3	40.7	10	2	11
	21	7–27°	0.29	3	36.7	11		
	19	12–32°	0.37	6	28.4	11		
	14	13–33°	0.39	9	27.0	11		
post.	12	16–36°	0.44	10	24.7	11	3	11
dist.	15	13–33°	0.39	18	21.6	9	3	9
	11	20–40°	0.50	11	19.8	10		
prox.	14	21–41°	0.52	6	20.6	11	4	8

length is rather constant. The calculated value of 10–11 μm was confirmed by some direct observations of λ from surface views of metachronal patterns. In the oral groove the average wave angles remained in a normal range, but calculated and observed values of wave length appeared to be somewhat smaller than on other regions of the body.

The data drawn from Table 1 allowed the construction of a generalized pattern of metachronal waves in forward swimming *Paramecium* (Fig. 2). This pattern, which was exclusively constructed from profile data with the assistance of model wave patterns, corresponds to well-known surface views of living and fixed metachronal waves in *Paramecium*.

Successful applicability of ciliary model patterns for the analysis of coordinated ciliary activity confirms the following conclusions:

Fig. 2. Generalized metachronal wave pattern of forward swimming *Paramecium* at normal conditions, calculated from side views of beating cilia (Table 1). Assumed body length: 250 μm

(1) The ciliary cycle in active paramecia under normal conditions is – in terms of Kuźnicki and coworkers – a conicoidal, counterclockwise gyration, but polarized in its temporal and spatial course and accompanied by periodic bending and straightening of the cilium.

(2) Populations of body cilia, if working at constant frequency, give rise to a dexioplectic metachronal wave pattern which usually proceeds in the anterior right direction.

(3) Wave patterns of instantaneously fixed paramecia correspond to natural conditions. Statements 1–3 were repeatedly expressed by Párducz (see 1967). Statement 3 recently was confirmed by Tamm and Horridge (1970) to be valid in *Opalina*.

(4) It is not possible to attribute different kinds of ciliary activity to stationary and free-swimming paramecia. The beat is more or less polarized in each case. Unpolarized conicoidal ciliary beating, as described by Kuźnicki and coworkers in stationary paramecia, was not seen in combination with metachronal waves; perhaps it may be existing in resting animals at very low levels of ciliary activity.

(5) Unpolarized helicoidal beating was not observed at conditions of normal viscosity. However, as can be seen in the model cilia, the polarized ciliary beat is always somewhat helicoidal, but pitch and diameter of the helix undergo enormous changes during the ciliary cycle.

(6) Profile views of metachronal waves leave metachronal pattern parameters undetermined. They only can show antiplectoid (=apparent antiplectic) or symplectoid (=apparent symplectic) coordination, depending on the angle of observation of the line of ciliary synchronization. Ciliary model waves help to reconstruct the true metachronal type.

It was shown that ciliary models may be useful for understanding of some basic properties of the polarized ciliary beat and its metachronal coordination. However, exact temporal dimensions of polarized ciliary activity can hardly be incorporated into a stiff model. Beat frequency and timing of the effective and recovery stroke, which depend on membrane potential, temperature and other physical factors such as viscosity of the liquid medium, must be described by quantitative methods of cinematographic and stroboscopic recording.

Summary

The spatial properties of the polarized ciliary beat in *Paramecium* were ascertained by a wire model of metachronal activity, which was constructed according to photographs of surface and side views of swimming paramecia. In body cilia the metachronal type is dexioplectic, whereas profiles of metachron waves because of their 2-dimensional nature show "antiplectoid" or "symplectoid" ciliary configurations. Wave length and wave angle can be inferred from profile views with the help of the ciliary model.

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EXPLANATION OF PLATES I–VI

1–3: Examples of ciliary activity in *Paramecium multimicronucleatum* under normal conditions (20°, lcP). (1) Forward swimming, (2) slightly compressed forward swimming, (3) slightly compressed stationary specimen. Vertical bars in these and in the following pictures indicate 10 µm
4: High power surface view of ciliary metachronal waves in a forward swimming *Paramecium*, anterior end. Numbers correspond to sequential stages of ciliary beat; compare model cilia in Pl. III 6
5: Micrograph of the normal ciliary pattern of metachrony in a fixed and stained *Paramecium caudatum*. Preparation embedded in Vestopal
6: Seven stages of model cilia according to the ciliary cycle in a metachronal wave of a forward swimming *Paramecium*. 1–3 – Effective stroke, 3–7 – recovery stroke. Wide arrow: direction of the effective stroke; narrow arrow: direction of metachrony
7: Model of metachronal ciliary activity in a forward swimming *Paramecium* at normal conditions. Proportions of cilia and cell cortex according to observations. Radiating lines around the model indicate defined planes of optical sections at right angles to the model surface. WF, wave front
8: Catalogue of profile views of the ciliary model. Numbers indicate angles between the line of synchrony (wave fronts) and the plane of optical focus. (a) Waves of body cilia; (b) waves of peristomal cilia
9–11: Comparison of some common ciliary profile views of forward swimming paramecia with model waves. (9) Cilia of posterior right body side of Pl. I 1. (10) Posterior dorsal body edge. (11) Detail of anterior right portion of Pl. I 2; note symplectoid configuration of the cilia (arrowhead). Numbers in the models indicate range of wave angles
12–13: Ciliary beat and wave form in the oral groove of forward swimming specimens. (12) Anterior end, ventral left. (13) Midregion, ventral; note reversed beat and wave direction in the peristome (arrows)
14: Comparison of the peristomal ciliary wave (Pl. VI 12) with a model wave profile at 20–40° wave angle

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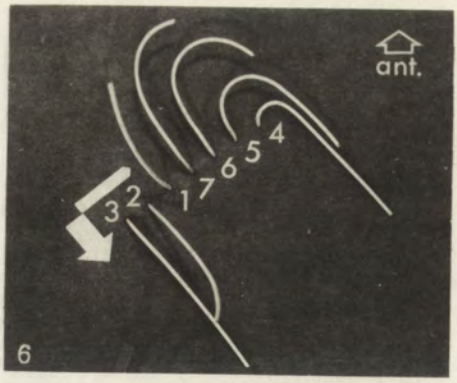

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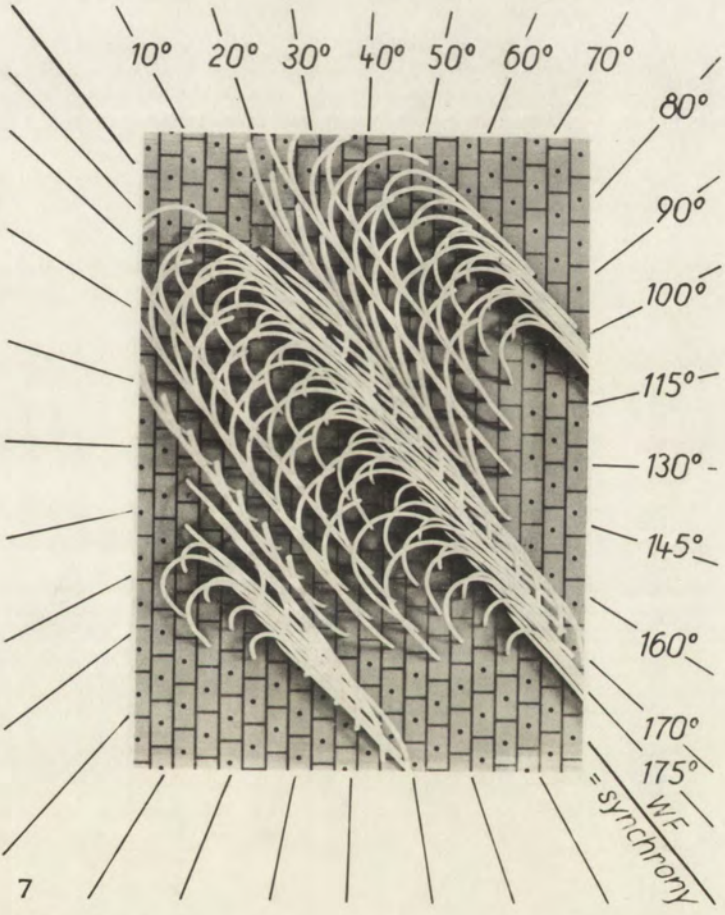

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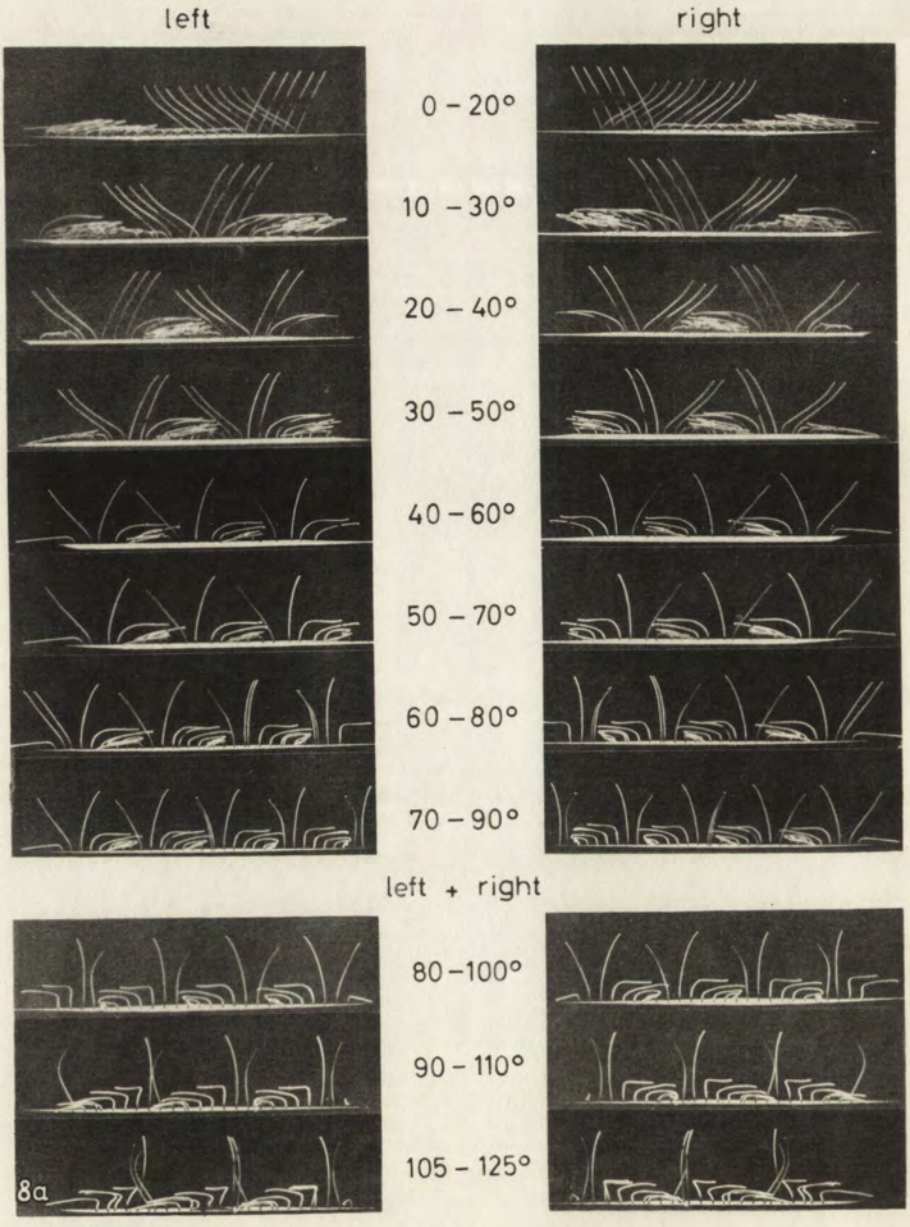

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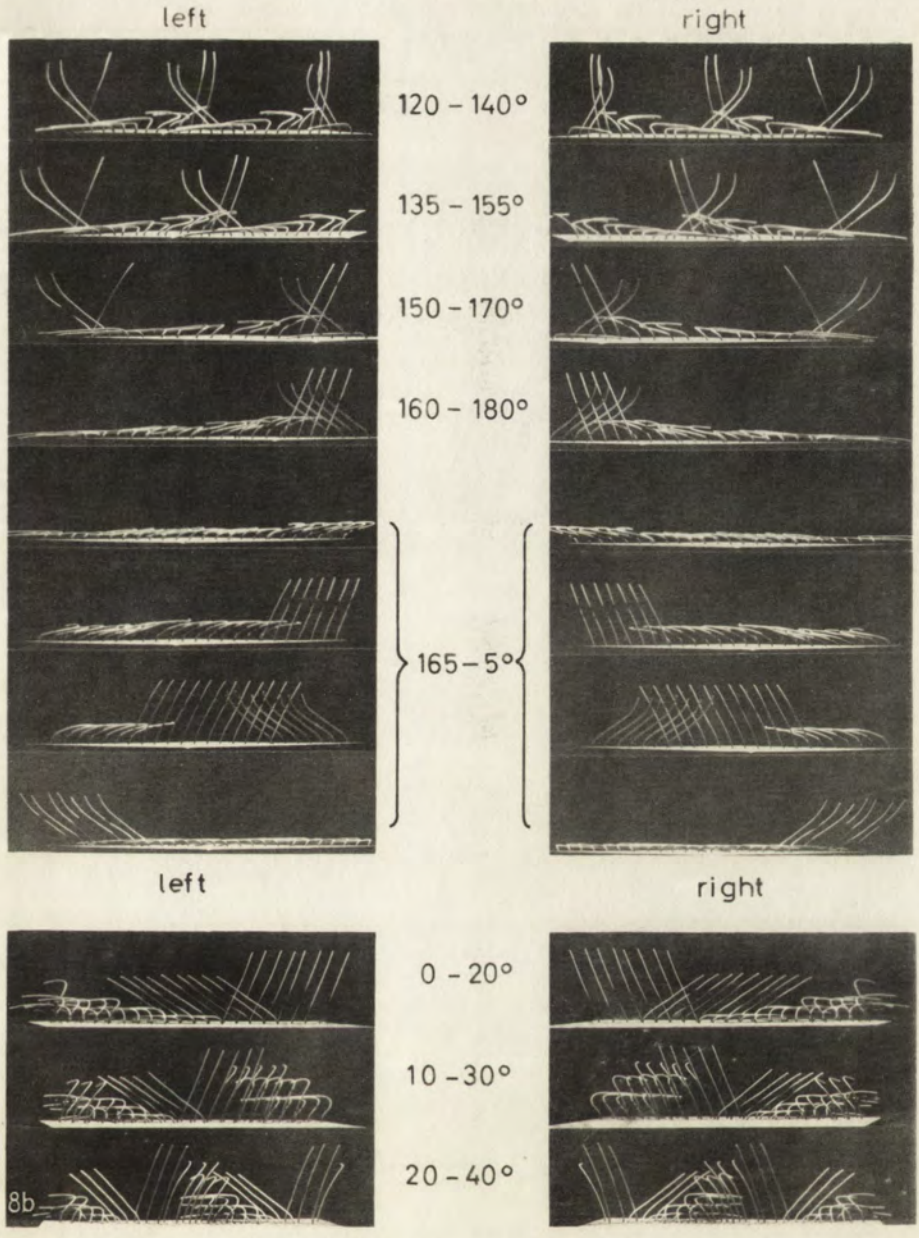

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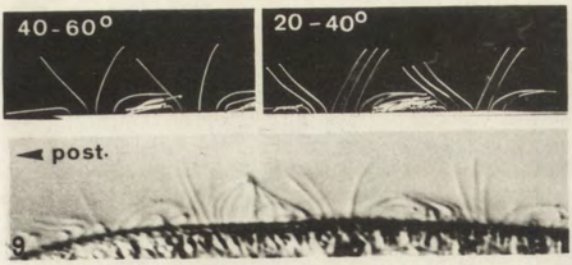

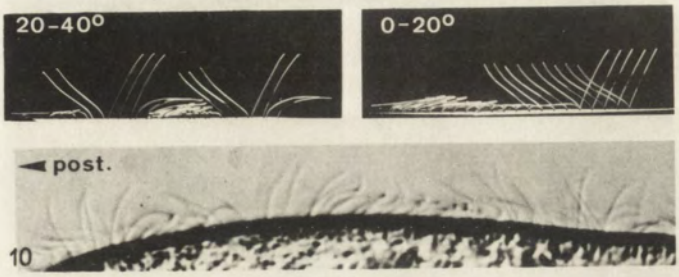

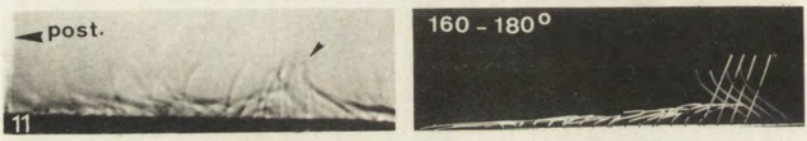

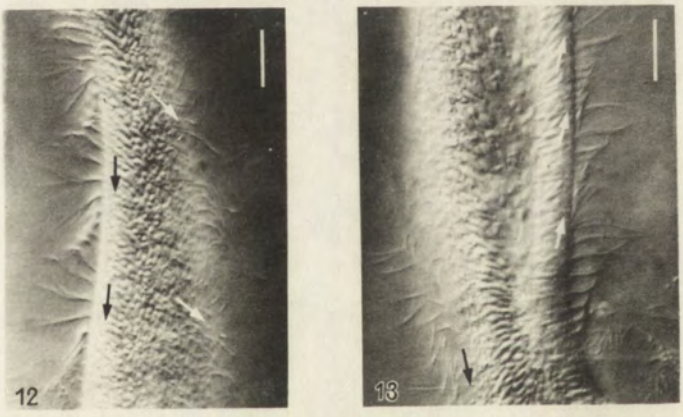

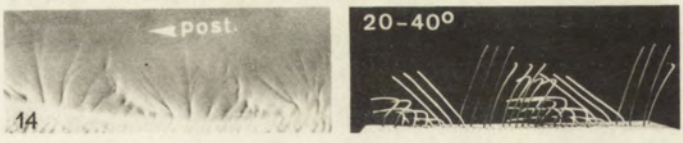

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Motile behaviour in two forms of double animals *Stylonychia mytilus*

Illustrated with 16 mm black-white motion picture

The double forms of *Stylonychia mytilus* differ from normal, single forms not only by their morphological features but also in their motile behaviour what might be of some importance for the experimental studies on function of various groups of cell locomotor organelles.

Stylonychia possess characteristic oval or kidney shape of the body with the flattened ventral and convex dorsal surface. The long and strong membranelles of AZM embrace the frontal part of animal from the right to left side and then from the left side it bows to the middle of the cell towards buccal cavity. Two membranelles undulans run from the region right of the buccal cativity to the front of animal. On the ventral side of the ciliate there are eighteen cirri which Stein (1859) divided into three groups: eight "frontal", five "ventral" and five "transversal". Recently Machemer 1965 distinguished only three "frontal" cirri and connected the remaining five frontal with five ventral ones into one big group of "ventral" cirri, while "transversal" cirri he called "caudal" ones.

On the left and right side of the body there are single rows of marginal cirri. The right row is longer and contains longer and stronger cirri than the left one (Fig. 1 A). On the dorsal surface of the body there are five rows of small dorsal bristles which are visible only in the argentophilic preparations. Near the second, third and fourth row of bristles on the posterior end of animal grow three long terminal cirri (Tchang et al. 1965, Frankel unpublished).

The double forms of *Stylonychia mytilus* may appear spontaneously in nature or they can be induced experimentally. At present we distinguish two forms:

(1) The "regular" or so-called "spherical" doublets which appeared spontaneously in culture as a result of disturbance during first cell division after conjugation (Totwen-Nowakowska 1964).

(2) The "irregular" or so-called "flat" doublets which were first described by

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Stein (1959), but were also induced experimentally by Tchang et al. 1964, Tchang and Pang (1965) after exposure of single forms to appropriate concentration of ethyl alcohol or by microsurgery operations of dividing cells.

It be emphasized that both forms of double animals could be induced by fifteen minutes lasting thermic shock (temperature in range 33–34°C) of dividing specimens of *Stylonychia* (Totwen-Nowakowska 1965, 1969), applying similar technique to that described by Zeuthen and Scherbaum (1954), Rasmussen and Zeuthen (1962) and Frankel (1962, 1964 a, b) for induction of synchrony of cell division in *Tetrahymena*. Both forms of double animals can be easy grown in laboratory conditions even for hundreds of generations (Totwen-Nowakowska 1969). However, they can transform back to single forms spontaneously. Observations described in this paper were made in Pringsheim's medium at temperature 22–24°C.

The morphology of *Stylonychia mytilus* doublets

"Spherical" doublet

"Spherical" doublet is formed by two identical partners joined together by their dorsal surfaces (Fig. 1 B). In both partners the shape of body, proportions and cirri systems are corresponding to the normal single individuals. Every part of the doublet has normal developed AZM with intact cytostome, normal set of all cirri. The nuclear apparatus is double.

"Flat" doublet

It is shown on the scheme drawing (Fig. 1 C) that this form consists of two partners connected side-long very strongly. The border line between them is hardly visible. The nuclear apparatus is double. The right partner is shifted posteriorly in relation to the left one. The ventral surface is flattened and the dorsal one is convex and all cirri of the ventral side are in the same plane. AZM and cytostome of the left partner are normally developed. The right partner is smaller than the left one and its AZM is shorter with reduced number of membranelles. Proportion between numbers of membranelles in both AZM-s is not constant. The arrangement of AZM and all other groups of locomotor organelles in the right partner shows the mirror symmetry in relation to the left one. Due to this fact the "flat" doublet is lacking completely one row of marginal cirri which corresponds to the right marginal cirri of single form of *Stylonychia*: consequently the marginal cirri of the right partner (i.e., the right row of marginal cirri of "flat" doublet) are similar in structure and function to those of the left partner.

All other cirri of ventral side in both partners come together in the middle region of body. It is impossible to mark out which cirri belong to the right and which to the left partner. So we can only distinguish two sub-groups of frontal cirri: Three

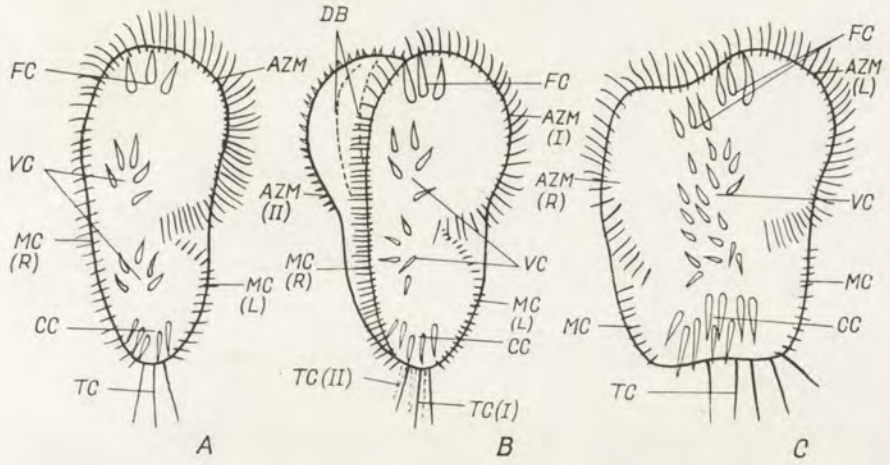

Fig. 1. Cortical anatomy of *Stylonychia mytilus*. A—normal, single form, B—"spherical" doublet, C—"flat" doublet, Abreviations: AZM—adoral zone of membranelles, FC—frontal cirri, VC—ventral cirri, CC—caudal cirri, TC—terminal cirri, MC—marginal cirri, (R)—right, (L)—left, DB—dorsal bristles

left frontal and little shifted backwards three (sometimes reduced to two or one only) right frontal. The number of ventral cirri may vary from fifteen to twenty two. They are thinner and smaller than frontal and caudal ones. The number of big, long and stiff caudal cirri fluctuates between nine to twelve, while the number of terminal cirri is between four to eight. The left marginal cirri run in similar way as in normal, single form of *Stylonychia*. The row of marginal cirri in the right partner is formed by smaller number of cirri than the left one.

Motile behaviour of *Stylonychia mytilus*

It was described elsewhere (Dryl 1965) that in normal single form of *Stylonychia* we can distinguish two principal forms of movement: (a) walking on the substrate and (b) free swimming in the fluid medium. *Stylonychia* runs or walks on the substrate due to action of all ventral and marginal cirri. During forward movement the direction of effective stroke of AZM and marginal cirri goes from front to back along the left and right edges of the body. Predomination of action of right marginal cirri causes the animal to move left-forwards along an arc. During reversal of cirri beat the action of right marginal ones causes the circling left-backwards movement (Fig. 2 A, B). Under normal conditions the short-lasting backward movement resulting from reversal of cirri beat appears as spontaneous movement or in response to various external stimuli.

Motile behaviour of "spherical" doublet

"Spherical" doublet posses complete double set of all locomotor organelles and consequently the animal swims in the free medium with higher speed and more frequently than single form. This may depend on the fact that in this form usually only one partner is attached to the substrate while the other one is exposed to liquid medium without any direct inhibition of locomotor apparatus by mechanical stimulation (Dryl and Totwen-Nowakowska 1966). Spontaneous reversals of cirri

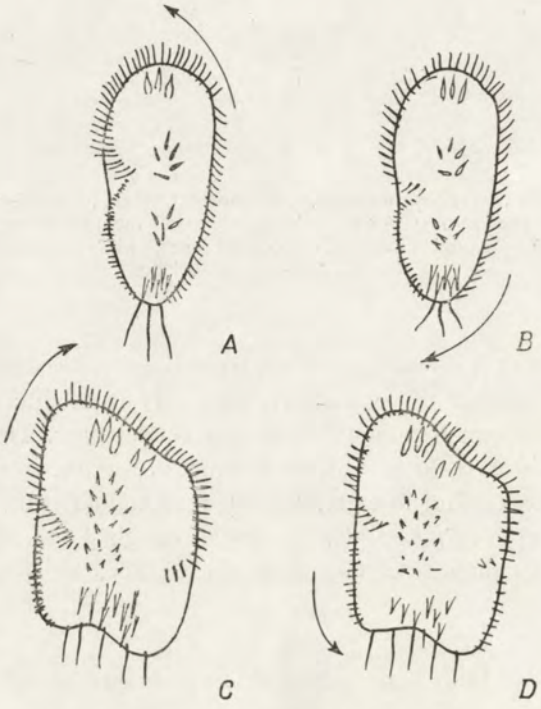

Fig. 2. Movement of *Stylonychia mytilus* (walking on the substrate). Single form (normal *Stylonychia*) (A) forward and (B) backward movement; irregular "flat" doublet. (C) forward, and (D) backward movement

beat occur also in this form of *Stylonychia* and they are often followed by shorter or longer periods of swimming in the liquid medium. Sometimes the partners are changing their positions, walking on the substrate alternately.

Motile behaviour of "flat" doublet

This form shows so completely pattern of movement from that of single and "spherical" form that it is hard to believe that it is derived from *Stylonychia*. We can call this form "thigmotactic" because – as a rule – it walking slowly on the

substrate and shows very often complete stopping of progressive movement. Swimming in the free medium is almost absent and the spontaneous reversals of cirri beat occur seldom. When moving forwards or backwards, the animal turns to the right as a result of stronger action of cirri in the left partner (Fig. 2 C, D).

Summary

The double forms of *Stylonychia mytilus* differ both in morphological features and motile behaviour. The so-called "spherical" doublet consists of two identical partners joined together by their dorsal sides; it possess a complete double set of locomotor organelles and is more actively swimming than the original single form. The so-called "flat" doublet consists of two partners joined together by their lateral sides. AZM and cirri of the right partner are reduced in number and show the mirror immage symmetry in relation to the left one. Due to this fact the animal is lacking completely one row of cirri which corresponds to right marginal cirri of normal, single form of *Stylonychia*. The "flat" doublet is swimming at very slow rate and walking on the substrate forwards or backwards along arcs with deviation to the right side as a consequence if strong action of AZM and cirri of the left partner.

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Motile and contractile activity in connection with the excitable process and the functional state of the cell

Contemporary cytophysiological science more frequently comes across the phenomenon when the complex vital activity of organism as a whole is regulated at the cellular level. In connection with this fact, investigation on protozoa assumes ever greater importance for general biology. A single cell which has been the phylogenetic and ontogenetic precursor of metazoa, should be the genetic carrier of its hereditary properties, shaped in the course of evolution (Bonner 1965).

The theory of life's origin counts off its existence from initial protobionts (Oparin 1968). This fact allows us to regard amoeboid animals as the immediate prototype of primary cell. At the early stages of living structures development there was the primitive irritability which gave rise to the specific excitability. There was also the contractility which was used afterwards as the basic for specific contractile structures of metazoa formations (Koshtoyants 1954). Both phenomena – irritability and contractility – came into being as the common properties of the same structure. However, at the unicellular stage of animal's development we could observe two types of contractile organells in a single cell: myonemes responsible for the contraction function of the cell, and motile organells – the cilia and the flagella. Unicellular organisms also have the formed boundary excitable structure.

The relatively stable character of intracellular texture in every living organism makes it possible to believe, that intracellular systems successfully shaped at the beginning, can be preserved at all stages of evolutionary development. Thanks to this, structural and functional protozoan properties give ground for understanding of a number of peculiarities of metazoan cells. With certain limitations these properties can be regarded as common for all living cells.

Three functional displays of cellular activity – cellular contraction, motility, caused by ciliary apparatus; and excitation in its classical sense will be discussed today using ciliates and opalina as an example. These functions will be discussed from the point of view of their possible interaction with one another. The first is

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cytoplasmic contractile ability which takes place in almost all protists. However, some ciliates have peculiar contractile machinery, for instance *Spirostomum ambiguum*. As a result of subpellicular contractile structures, deformation its normal form stops to be elongated and becomes ellipsoid (Fig. 1). By contraction the longitudinal axis of the cell becomes from 4 to 6 times shorter.

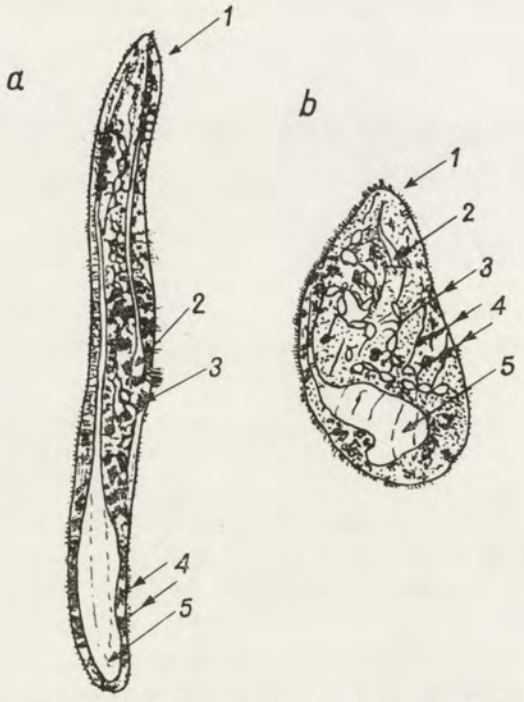

Fig. 1. Changes in the cell's form of *Spirostomum* by contraction: a—before contraction, b—during contraction, 1–5—cellular organelles

If we microfilm the contractile response of a *Spirostomum* to vibration and electrical stimulation and check the sequences for the magnitude of longitudinal cellular axis we shall be able to reproduce the different contraction phases. Calculations have shown[1] that the latent period measured as an interval between the moment of the stimulus and the reaction averaged 60 msec (variations for different cells can ranged from 30 to 120 msec). The contractile phase averaged 50 msec. Relaxation to the initial level: 3–4 sec (Fig. 2). No considerable changes in the contractile curve and in the length of the latent period were observed when the K, Na, Ca contents in a cultural medium increased 2–3 times. The displacement affected the relaxation curve only and remained within the possible variations of the control curve. The Na^+ and Ca^{2+} delay the relaxation time towards its highest value (4 sec), whereas

[1] The calculation of curves in Fig. 2 was carried out by A. Galkin.

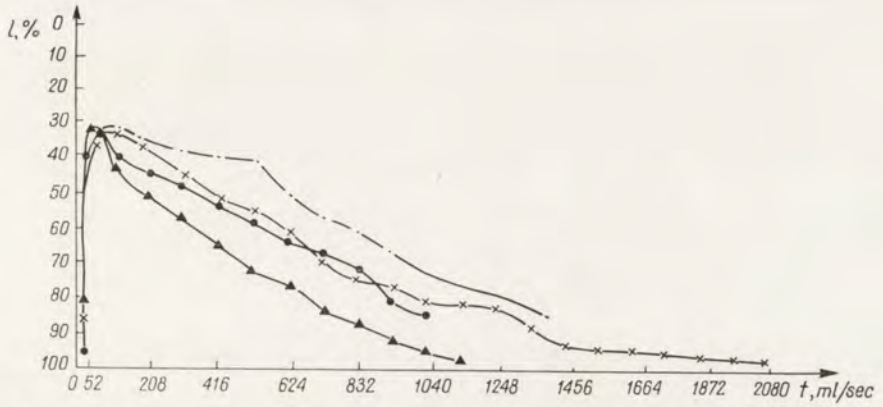

Fig. 2. The contraction curves of *Spirostomum* in culture medium and in media having a surplus of KCl, NaCl and $CaCl_2$. Across the vertical line—the length of contracted cell in percent to the initial length. Across the horizontal line—the time in ml/sec. ▲—▲—▲ K^+ (0.54 mM KCl), × — × — × Na^+ (20 mM NaCl), · — · — · Ca^{++} (50 mM $CaCl_2$), ● — ● — ● control

K^+ towards its lowest value (2 sec). Characteristic for the action of Ca -rich solution is the emergence of a plateau at the beginning of the relaxation phase. The threshold of contractile reactions also undergo some changes: they increase in $CaCl_2$-rich solution and decrease in KCl-rich solution.

Thus our date demonstrate a relatively weak influence of ionic compounds of the external medium on the contractile act itself.

The motoric function of a ciliate consists of complicated behavioural reactions: forward and backward motion, rotation around different axes of the cell body. These mobile acts proceed from distinctions between the beating direction of various cilia and the time sequences in their beats. The temporal and spacial factors provide the pattern of the metachronal wave (Fig. 3 a). Just these waves and not the contractile act of every cilium itself provide the forms of cell mobility. Instant fixation of *Opalina ranarum* cells using the Paraducz method (1953) permits to fix the methachronal wave's pattern. Numerous data provided by different authors attest to the great ionic sensitivity of cilia (Seravin 1967). As our experiments show, K^+ in rather high concentration (5 mM KCl) provokes a general reversal of the beats of all the cilia of the *Paramecium caudatum*. A small predominance of this ion in cultural medium, i.e. when K^+/Ca^{++} equilibrium displaces toward K^+ (no − Ca solution) causes numerous local reversals of small ciliary groups. The general pattern of the metachronal wave breaks up into separate small waves. This can be seen clearly in *Opalina ranarum* (Fig. 3 b). Our observations carried out with Doronin, indicate that similar local reversals occur with *Spirostomum* in K^+-rich (2.7 mM KCl) and Na^+-rich (2.9–20 mM NaCl) media. Increasing numbers of "search" reactions consisting of short jerks in different directions were an external manifestation of local reversal. Motile activity of the *Spirostomum* in ordinary cultural medium and in monovalent cation − rich medium was recorded (Fig. 4). In the first case cellular

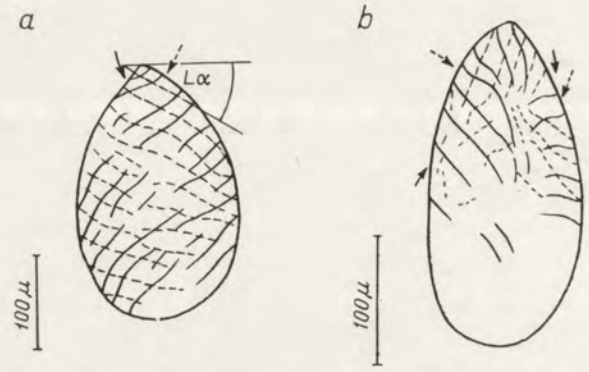

Fig. 3. Metachronal waves of *Opalina ranarum*. a—in ordinary salt solution (Lα—is the angle between the perpendicular on longitudinal axis of the cell and the line of metachronal wave, b—in KCl surplus medium. Traced with Camera lucida. Stained with Heidenhain haematoxylin method. The arrows indicate the lines of the waves. Solid lines—"dorsal" waves, dotted lines—ventral waves. Cellular orientation according to Kinosita (1954)

motion was recorded in the form of separate deep waves (Fig. 4 a). Its amplitude was determined mostly by the size of the chamber in which the infusoria were swimming. The recorded picture changed under the influence of sodium and potassium. Small and low amplitude fluctuations became predominant (Fig. 4 b). As our data showed, the same local reversal by acetylcholine in concentration 5×10^{-5} and by

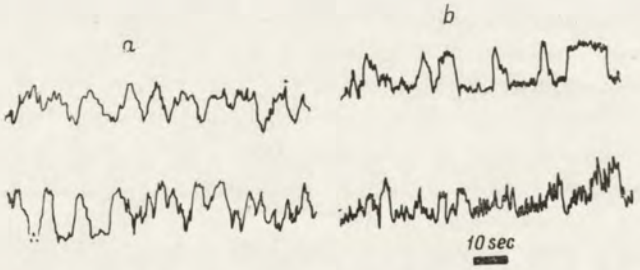

Fig. 4. Motile activity of *Spirostomum*: a—in culture medium, b—in KCl surplus medium (20 mM). Kymographic registration was made by means of sighting device in the form of two components (upper and lower curves)

anticholinesterase substances might be provoked. All these drugs were able to induce both general and local reversals. Calcium did not exert direct influence on the metachronal wave pattern. Nevertheless it counteracted the ability of potassium for causing local reversals (Kokina 1960). The above results of our experiments and those of other authors attest to the great sensitivity of methachronal wave's pattern to ionic compound of environment.

Since the contractile and mobile functions of the cell are effectoral, the question

arises about their relationships with a third functional display of the cell i.e., its excitability. The modern membrane theory determines cellular excitability as a capacity for specific rapid alteration of ionic permeability in response to external stimulation, accompanied by active electrogenesis (Khodorov 1969).

The electrical characteristics of the superficial cellular layer, its specific electrogenesis caused by the certain factors side by side with other parameters can be used as reliable criteria of excitability. As stated in our previos papers (Kokina 1965, Kokina and Doronin 1970) the properties of a number of electrical protozoan parameters and their sensitivity to neuropharamacological drugs allow us to consider the formation of the common excitable laws and the excitable cell functions on a single-cell level of living structures development. Proceeding from what has been said above a resting potential (RP) of the cell and its electrogenesis have been chosen as criteria of excitable properties. The species RP – individuality must be noted first of all when giving its characteristics. Thus in *Opalina ranarum* RP has an constant sign and reveals a true ionic selectivity to potassium. Its electrogenesis is represented on the whole by rhythmical electrical changes of RP, within 10–20 mV. The basic peculiarity of infusoria *Spirostomum ambiguum* is the magnitude and sign plurality of its RP. The character of this diversity is evidently dependent on ionic environmental composition. In ordinary cultural medium RP has a positive sign and its average value amounts to +36 mV (changing within 31–47 mV), (Fig. 5 a). Its terminal level by two or more steps may often be reached (Fig. 5 b, c). Side by side with positive RP there are negative RP both in cultural media and pure salt solutions. (Fig. 5 d, e). Statistic analysis of the common totality of *Spirostomum* RP has made it possible to determine a series of real RP – levels both positive and negative (Fig. 6). In some experiments the concentrations of KCl, NaCl, $CaCl_2$, $MgCl_2$ in surrounding media were modified from 0.1 mM to 100 mM. It turned out that at lower ionic strength positive RP were rather frequent. Increasing concentration of bivalent cations in the medium increased the number of negative RP. Mainly negative RP were measured in 10 mM $CaCl_2$ and $MgCl_2$ solution. When KCl, K_2SO_4 and NaCl concentration was raised the number of positive RP was reduced. In some cases noteworthy is a mirror semblance of recorded RP of both signs. The magnitudes of RP from different signs were similar. For example: in 1 mM $CaCl_2$ – medium we recorded RP +37 mV and – 37 mV; +14 mV and –14 mV, etc.

The present observation perhaps suggests the existence of a complex electrical structure in *Spirostomum*. But our morphological knowledge and even electronmicroscopic study do not make it possible to draw morphological parallels. The above mentioned diversity makes it difficult to apply a membrane theory for explanating their nature. RP values calculated with the help of Nernst's equation[2] may approximate the maximal experimental values only within a narrow concen-

[2] Intracellular ionic concentrations were taken from Carter's paper (1957).

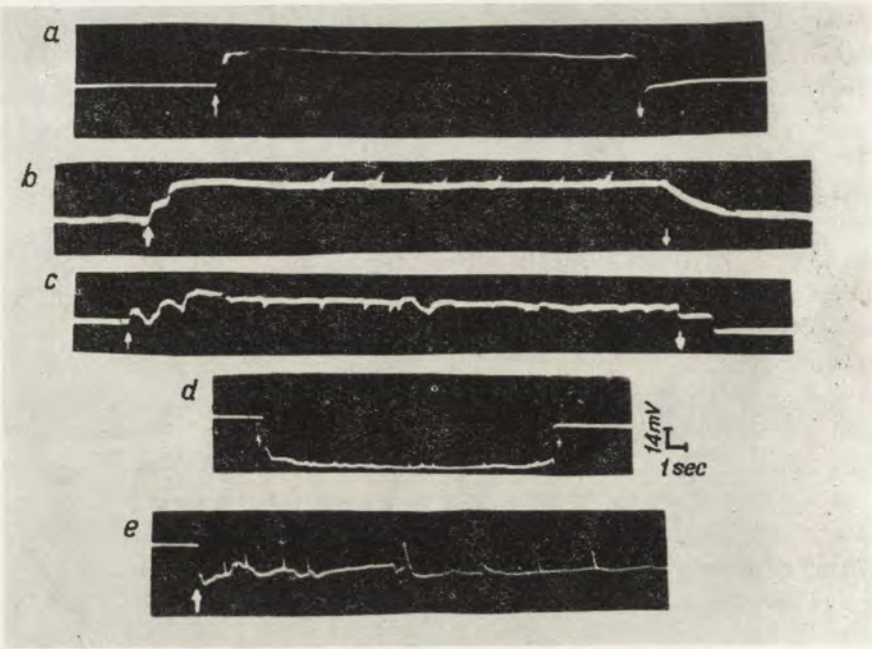

Fig. 5. Intracellular potentials of *Spirostomum ambiguum*: a, b, c—in culture medium (KCl—4 mg/l, NaCl—30 mg/l, $CaCl_2 \times 2H_2O$—5 mg/l, $MgCl_2 \times 7\ H_2O$—5 mg/l, $NaHCO_3$—7.5 mg/l), d—in $MgCl_2$ surplus medium, e—in $CaCl_2$ surplus medium. The arrows indicate input and output of the intracellular microelectrode. Contractions of the cell without potential changes are ticked off on oscillogramme "b". The "c, d, e"—tape-recordings demonstrate some rhytmic changes of the RP

tration range. Thus, for example, RP in a low salted media of low ionic concentrations was closer to Cl − gradient calculated value. Experiments carried out by other authors on *Amoeba* (Batueva 1968), Cilliates (Dryl 1970), Flagellates (Chang 1960) have also revealed the individuality of RP.

Besides RP its slow discharge with the following parameters may be observed in *Spirostomum*, namely electrical oscillations with 100–1000 msec duration and 1–10 mV amplitude, assymmetric in form (Fig. 5 c, d, e). The falling phase is at least 7–10 times longer that the rising phase. The polarity of these potential changes sometimes differs. RP of both signs occur with both depolarizing and hyperpolarizing oscillations. Since these potentials are usually accompanied by cell contractions and repeat contractile rhythm, their possible connection with the contractile act has been discussed. Our subsequent experiments have convinced us of the absence of direct correlation between RP-alterations and contractions in *Spirostomum*. We recorded contractions without potential changes (Fig. 5 b) and oscillations without contraction (Fig. 7 a, b).

In subsequent experiments we artificially removed RP by direct current flow through a two-barrage microelectrode. When this way of voltage feeding into the

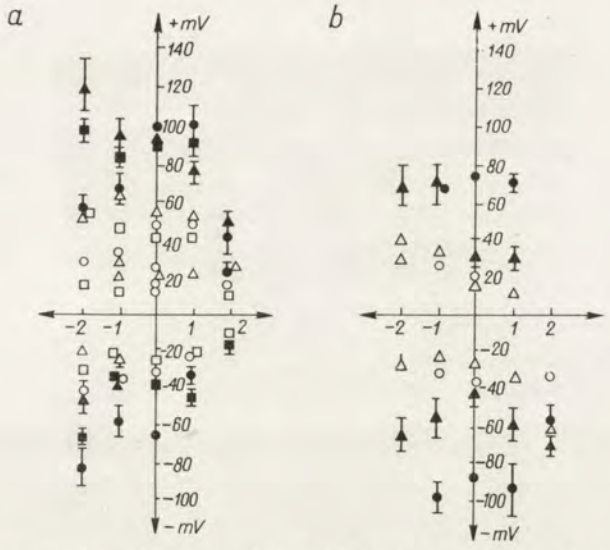

Fig. 6. Resting potential in different solution (*Spirostomum ambiguum*), a — ● ● — maximal values in KCl, ▲ — maximal values in K_2SO_4 ■ — maximal values in NaCl, ○△□ corresponding average of RP and reliable RP levels ($p \geqslant 0.95$) b–▲— maximal values in $CaCl_2$, ● — $MgCl_2$, △○ the average in $CaCl_2$ and $MgCl_2$ solutions. On the abscissa logarithm of salt concentration. On the ordinate — RP in mV. Measuring of RP and treatment of results were made by Ju. Doronin

cell was carried out no myonemes contraction was obtained. Contractile reaction by intracellular impulsive electric stimulation was also absent. Stimulating impulses of both polarity were applied. There characteristics varied on a large scale: frequency 1–30 Hz, stimulus duration 0.5 msec–3 msec., voltage of the output of capacitor 1–15 mV.

It appeared possible to assume the mechanical-artefact nature of recorded oscillations. One could suppose that they resulted from changes of microelectrode shunted resistance. The thickening of cytoplasm around the tip of the microelectrode can be expected to cause its increase at a contraction act. At the same time when the cell came off the microelectrode shunted resistence may decrease. That these artefacts may exists is clear for anyone who has to do with intracellular microelectrode recording from moving objects.

Subsequently these suggestions were safely rejected since the active variations in potential differences of *Spirostomum* contained without contractions. Intracellular stimulation, non-effective with respect to contractile reaction, caused ciliary reversal and negative electrical response (Fig. 7 a). This response entailed the replacement of RP value on a new level, which was maintained throughout the period of stimulation and reversal. Cutting off stimulation current was followed by a progressive restoration of the initial RP level. Described reaction appeared only at a sufficient frequency of stimulation discharge (10 Hz) and when inward current flow was applied.

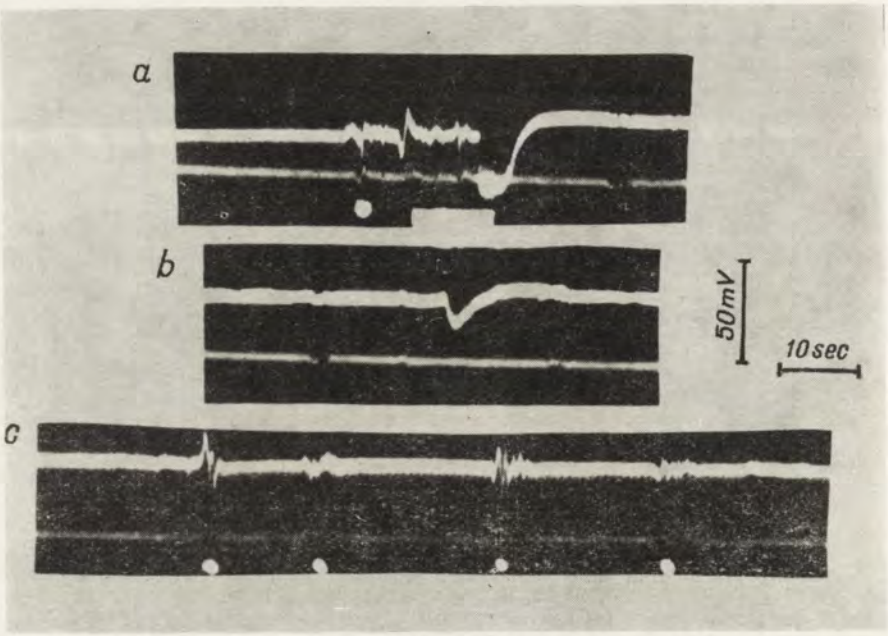

Fig. 7. Intracellular stimulation and registration of RP in *Spirostomum* with a two-barrel microelectrode, a—an active negative reaction produced by prolonged stimulation. The frequency of stimuli 10 Hz, duration—1 msec, the intensity on the output of the stimulator equals 2 V. The black line indicates the time of stimulation, b—spontaneous electrical changes of the same type. In both cases (a, b) cell contraction are absent. c—the absence of active electric response after the replacement of current polarity from negative to positive. Artefacts obtained from switching the current are marked with dots. On every tape-recording the upper line—represent potential from tapping electrode, the lower line represents potential from current-electrod

There is good evidence about the connection between RP and ciliary reversal in *Opalina*. Momentary cell fixation on the tip of the microelectrode allowed to compare RP – value with metachronal wave pattern. There is a direct relation between the electric potential and the angle formed by the wave's front and the perpendicular line on the longitudinal axis of the cell (Fig. 8). An adjusting action of RP on the beating direction of the cilia exists within a narrow potential range (−10)-(−35) mV. Beyond it the fluctuations of RP and ciliary waves commence. In *Spirostomum* the regulation act of RP is revealed at (+45)-(+15) mV.

Summarizing the experiments carried out with *Spirostomum* we came to conclusion that within one cell there are two autonomous systems of different functions: contractile and motile. The autonomy of contractile and motile reactions was revealed with particular clarity in their responses to electric stimulation, to the action of ionic factors and to the Rp – level changes.

When extracellularly stimulated by electric current both systems had a different threshold: 2 mV for cilia and 4 mV for myonemes. It is worth noting that contractile reaction at superthreshold stimuli are very irregular as distinct to the always regular

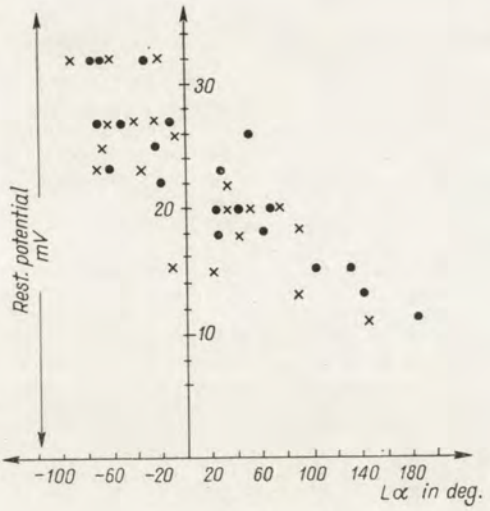

Fig. 8. Correlation between the value of RP and metachronal waves pattern of *Opalina*. On the abscissa – α in degree (in foremost part of cell body). On the ordinate – RP in mV at cell's fixation moment. × – "dorsal" side, ● – "ventral" side

ciliary response. The contractile act may be discharged in time relative to the ciliary response to the same stimulus. Data on hand, make it possible to put forward the hypothesis that the contractile reaction of *Spirostomum* to the electric stimulation is not direct and has an intermediate element. Ionic displacements in cytoplasma by assumed as mediatory factors (Sleigh 1970). The above-mentioned facts make it possible to say that unicellular protozoa give an example of the functional regularity important for single cell life origin.

The surface layer of the cell is the first one to be affected by environmental forces. It has different connections with contractile organells of the cell which accomplish different functions.

This heterogeneity in external stimuli perception gives to unicellular organism new abilities in analysing the environment.

Summary

Using two protozoa, *Opalina ranarum* and *Spirostomum ambiguum* as an example the authors were analysing the relations between the cellular resting potential (RP) and bioelectrical phenomena on the one hand, and the contractile and motile functions of the same cell on the other hand. It appears that RP of protozoa is directly connected with the metachronal wave's pattern and regulates the ciliary movement. At the same time it appears that there is no direct influence on the part of RP on the cellular myonemes contraction. This functional division of two contractile systems of a single cell (the myoneme system and the ciliary system) according to their interaction with immediate excitable cell structures is a definite evolutionary stage in the complication of integrative cell's activity.

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Patterns of body movements of *Euglena gracilis*

Since 1852, when Perty had described the body movement (the euglenoid movement, metaboly) of *Eutreptia*, many authors were interested in this phenomenon (Dangeard 1902, Beauchamp 1911, Günther 1927, Kamiya 1939, Conrad 1940, Chu 1946, Pringsheim 1948, 1956, Pringsheim and Hovasse 1950, Chen 1950, Hein 1953, Hilmbauer 1954, Diskus 1956, Fritsch 1961, Lozina Lozinsky and Zaar 1963, Mignot 1965, 1966, Leedale 1966, Arnott and Walne 1966).

Euglenoid movements of *Euglena gracilis*, the most common experimental model, have not been studied in detail. Fragmentary informations were given by Alexander 1931, Chadefaud and Provasoli 1939, Mackinnon and Hawes 1961 only.

In presented paper influence of the mechanical factors stimulating the body movement of *Euglena gracilis* had been studied. The purpose of the present work is to investigate:

(a) What main categories of body movement can be distinguished in *Euglena gracilis*?

(b) What is the biological significance of euglenoid movements?

(c) Whether the euglenoid movements could be induced by the mechanical factors acting directly on the cell body, or indirectly through the flagellum?

Material and methods

Euglena gracilis strain Z was grown in liquid pea medium. The experiments were carried out at 18–20°C. The behaviour of euglena was observed under light microscope and recorded by 16 mm Pentaflex cinematographic camera on colour UK 16 ORWO film. The motion picture were taken at rate between 0.8–32 frames per sec. The various changes of the shape were analyzed by means of film analyzing projector.

Euglenoid movements were induced by putting the specimen into:

(a) glass capillary (20–60 μ), to limit the free swimming space,

(b) methyl cellulose solution—to retard flagellar movement, or

(c) on the agar surface,

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

(d) between two slides with increasing pressure gradually.

Each type of experiment was previously performed on no less than 100 specimens. Behaviour of 20 organisms was recorded cinematographically.

Results

Euglena gracilis kept inside a wide capillary of 60 μ diameter, exhibits euglenoid movements rarely as freely swimming specimen in culture medium.

Inside narrow capillary of diameter smaller than 50 μ, flagellum from time to time touches the internal capillary walls, and euglenoid movements appear often. *Euglena* has enough space to contract and to assume a spherical disk-like shape (Fig. 1), with long axis of organism shorter than perpendicular one. Disk shaped

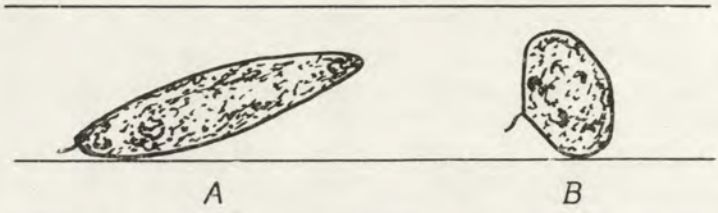

Fig. 1. *Euglena* inside a wide capillary (diameter 60 μ). A – elongate form, B – contracted body to a disk-like shape

specimens rotate and in consequence change very often the direction of locomotion. The same kind of body movements could be observed inside of wide capillary, but they appeared less frequently.

Further decreasing of capillary diameter up to 30 μ causes different kind of euglenoid movements. The contraction appears at the anterior part of the body and at first runs asymmetrically along one side of the cell. The anterior end turns down toward the place on the capillary surface which was touched by the flagellum. Then, contraction spreads on the whole body of euglena. The specimen rounds up, becoming spherical in shape. While euglena keeps this shape, flagellum actively pulls anterior end under the contracted body and finally appears on the other side of the capillary (Pl. I 1–21, Fig. 2 A).

During the contraction anterior end becomes little thiner and keeps its shape, until the body assumes a sphere (Fig. 2 C). At the same time posterior end becomes thicker (Fig. 2 D).

Relaxation also starts at the anterior end and than gradually spreads on the whole body of euglena (Fig. 2 B). As a result of those movements, body turns away of 180°. In the same condition (30 μ capillary) euglena which lost the locomotory flagellum occupies the whole space of the capillary, exhibiting slight, symmetrical contractions only.

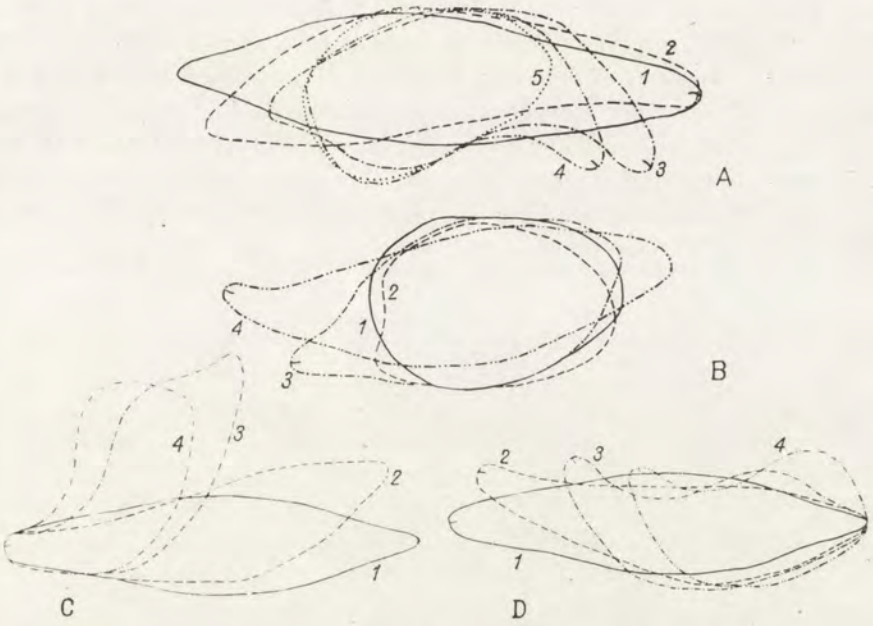

Fig. 2. The consecutive stages of asymmetrical contraction inside capillary (diameter 30 μ). A—consecutive stages of contraction, B—consecutive stages of relaxation, C—changes of the shape of the anterior end during contraction, D—changes of the shape of the posterior end during contraction

Similar behaviour of euglena could be observed inside of a very narrow capillary (diameter of capillary equal to specimen thickness) (Pl. II 22–29). The symmetrical contraction could be partial, that means only at anterior either posterior part of the body (Pl. II 22–25), or at both ends simultaneously (Pl. II 25–29). Obtained results show that:

(1) Frequency of euglenoid movement increases if diameter of capillary decreases.

(2) The flagellum is actively involved in changing of body position especially during turning away.

To establish the influence of higher viscosity on the euglenoid movement, euglena was put into methyl cellulose solution. The purpose of this experiment was to examine, whether the euglenoid movements could be induced by stimulation of flagellum only. The question is whether the flagellum may transmit the stimulus to the cortical layer of the cell and trigger the mechanism of euglenoid movement phenomenon.

As a rule euglenoid movement appears in 1% methyl cellulose solution. This indicates that flagellum is the receptor which transmits the stimuli of the body contractions. Euglenoid movements appear as a slight or strong contractions of the body.

Pressure of cover glass on the euglena body or gradually decreasing of the water

layer with specimens on the agar surface, causes, (besided induction of slight contraction of anterior or posterior end) the waves of contractions which run rhythmically along the long axis of the body. This kind of contraction is generated at the posterior end of the cell and moves toward anterior end. *Euglena* seems to be squeezed by the ring of contraction passing gradually along the body (Pl. III 30–37, IV 38–73, Fig. 3). New contraction generates when the previous one reaches the middle part of the body. The whole cycle of one contraction lasts 10–13 sec, while the new one appears usually after 6 sec.

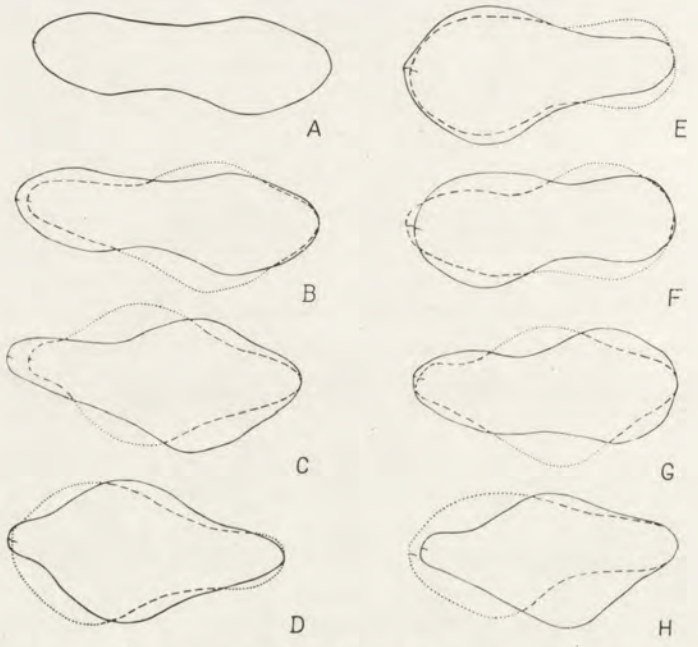

Fig. 3. Waves of contraction running rhythmically along euglena body. Initial shape drown by continuous line. The discontinuous lines were used for expressing stages of contractions. The region of contraction indicated with broken line, region of relaxation indicated with dotted line

More strongly pressed euglena (or when the layer of water on the agar becomes thin) is able to assume a shape of flattened sphere only (Pl. V 74–83, Fig. 4). Contraction and relaxation of the body runs regularly. The renormalization of the body shape starts at its anterior end causing pear-like appearance of organism.

The transition from elongate normal shape of the body to the flattened sphere takes 4–10 sec.

The "complete" contraction of body is often preceded by the slight one. After this euglena returns to its elongate shape (Pl. VI 84–86, Fig. 5 A) and than strongly contracts getting a disk or flattened sphere shape (Pl. VI 87–91, Fig. 5 D). During

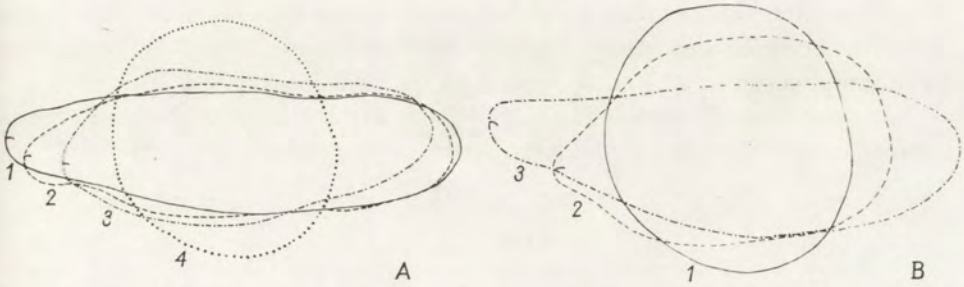

Fig. 4. Consecutive stages of contraction to a flattened sphere. A—successive stages of contraction, B—successive stages of relaxation

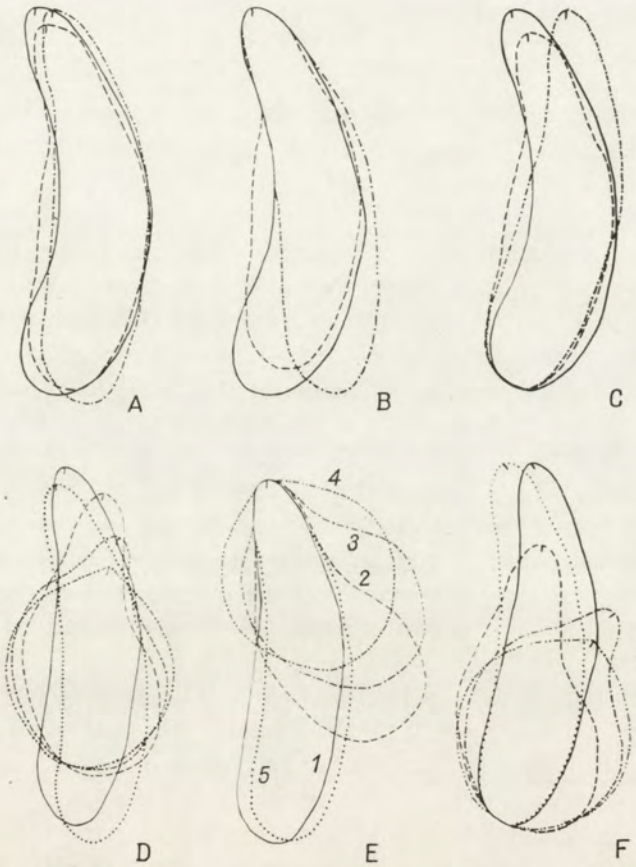

Fig. 5. Consecutive stages of slight and following it "complete" contraction. A—consecutive stages of slight contraction and recover to an elongate shape, B—changes of the shape of the anterior end, C—changes of the shape of the posterior end, D—consecutive stages of "complete" contraction and relaxation, E—changes of the shape of the anterior end during "complete" contraction, F—changes of the shape of the posterior end during "complete" contraction. Particular stages are designated as follows: 1 ———, 2 — — —, 3 —·—, 4 —··—, 5 · · · · ·

the slight contraction the anterior end does not change its shape (Fig. 5 B). The middle part of the body becomes little thicker (Fig. 5 C). During the "complete" contraction anterior and posterior area of the body get a sphere shape (Fig. 5 E, F).

The "complete" contraction may appear also in the case, when body of euglena was not completely relaxed after previous contraction (Pl. VII 92–99).

Discussion

Analysis of behaviour of *Euglena gracilis* under influence of mechanical factors, allows to distinguish the following patterns of euglenoid movements:

(A) symmetrical to the long axis of the cell,

(B) asymmetrical to the long axis of organism,

(C) appearing as a rhythmical waves of contractions running from the posterior to the anterior end of the cell.

The patterns of euglenoid movements are closely correlated with the external conditions. In the case of pattern A and B euglena is forced to choice such kind of body movement which would be the most effective for change of direction of swimming.

By means of rhythmical waves of contraction (pattern C) euglena can not change its direction but only slightly moves the posterior end forward.

So in case of *E. gracilis*, euglenoid movement could be involved in some kind of avoiding reaction and this confirms the previous supposition of Alexander 1931. The role of this movements would be the same as in *Peranema trichophorum*, which is able to change its direction of gliding movement only by contractions of the body (Chen 1950).

Probably in the other flagellates, the role of euglenoid movements in locomotion is more important. In non-flagellate species i.e., *Euglena leucops* or *Astasia captiva* and in the animals which commonly discard locomotory flagellum i.e., *Euglena mutabilis* or *Euglena deses*, the changes of the cell shape allow them to move in the surrounding medium (Beauchamp 1911, Hall 1931, Hein 1953, Mackinnon and Hawes 1961).

Observations and analysis of the behaviour of euglena body suggest that euglenoid movement appears under the directly influence of mechanical stimuli on the body or indirectly through the flagellum. As an example of the first case are the waves of contractions, which generate at the posterior end of the body. This observations confirm results Jahn and Bovee 1968 study. They observed that local pressure on the body of some species of *Euglena* normally capable of performing euglenoid movement would stimulate a local or general contraction of the body, depending on the amount of force exerted. The anterior end is the more sensitive area in this respect. On the other hand, occurrence of euglenoid movement in the higher viscosity solution (when the flagellum is the receptor of changes of viscosity) shows that

stimulus has to be transmited to structure responsible for body movements throughout the flagellum. Furthermore, experiments with euglena inside capillary show that flagellum actively collaborates with euglenoid movement in changing of body position. The connection between euglenoid movement and flagellum activity remains open question.

Summary

(1) Analysis of euglenoid movements of *Euglena gracilis* under influence of mechanical factors, has allowed to distinguish the following patterns of body movements:

(a) symmetrical to the long axis of cell,

(b) asymmetrical to the long axis of cell,

(c) rhythmical waves of contractions which run from the posterior to the anterior end of the cell.

(2) The distinguished patterns of euglenoid movements are interpretated as types of avoiding reaction.

(3) Euglenoid movements in *Euglena gracilis* do not play important role in locomotory functions but permit organism to change the direction of locomotion.

(4) The stimulus inducing euglenoid movements can be received directly by the surface or indirectly through the flagellum.

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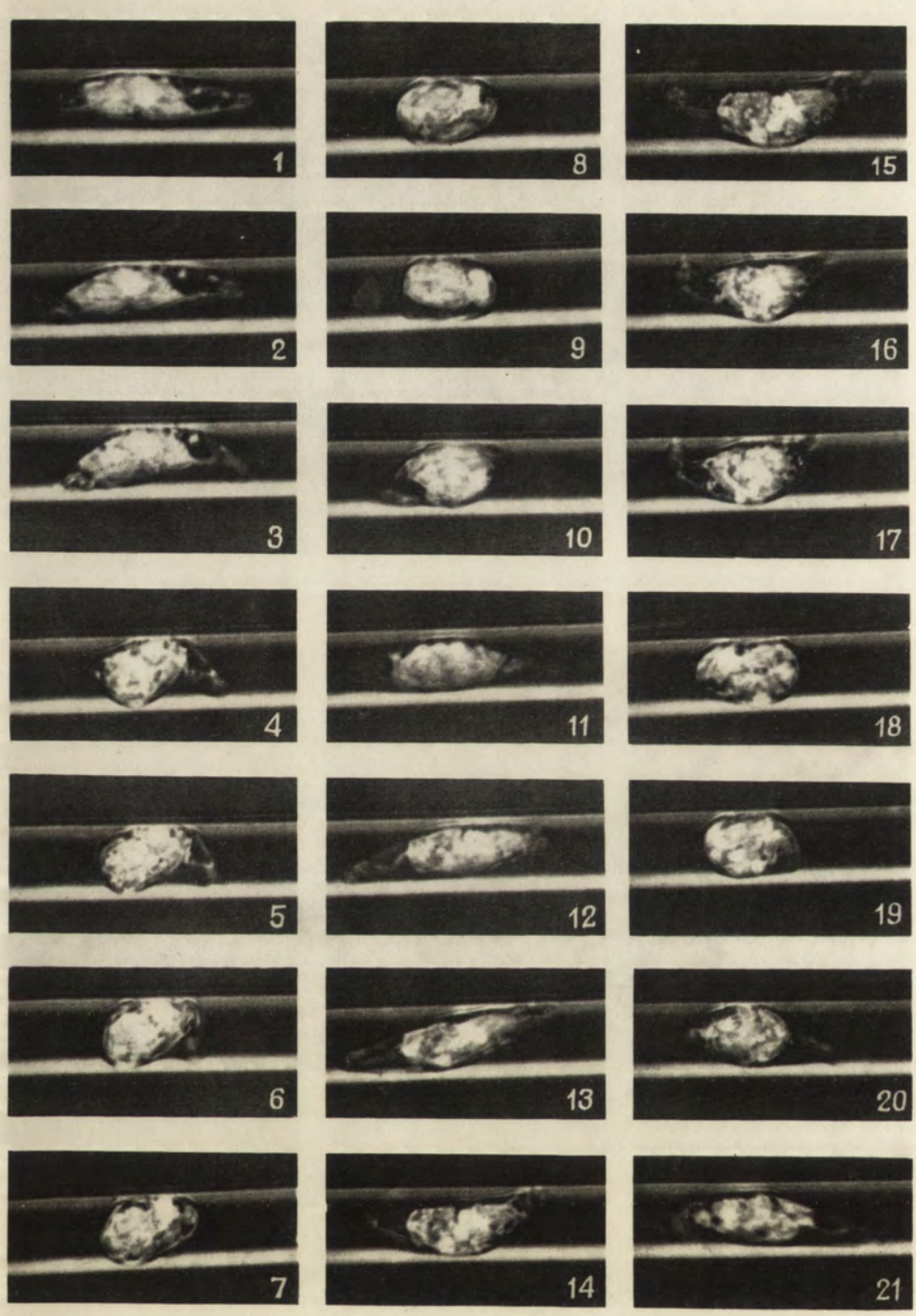
EXPLANATION OF PLATES I–VII

I: Consecutive stages of turning *Euglena* away inside of capillary (1–21)
II: Partial contractions of the body inside capillary. 22–25 – contraction of the anterior end, 26–29 – contraction of both ends simultaneously
III: Consecutive stages of the wave of contraction which runs from the posterior to anterior end under the influence of pressure of cover glass (30–37)
IV: Successive stages of four consecutive contractions. 38–46 – course of first contraction which reaches a middle part of the body, 47–55 – stages of the second contraction, 56–64 – consecutive stages of third contraction, 65–73 – stages of fourth contraction
V: Successive stages of contraction to flattened sphere and relaxation. 74–79 – contraction of the body, 80–83 – relaxation of the cell
VI: Slight and following it "complete" contraction to a shape of flattened sphere. 84 – elongate form, 85 – slightly contracted body, 86 – elongate form, 87–90 – contraction to a shape of flattened sphere, 91 – elongate form after relaxation
VII: Consecutive stages of "complete" contraction followed by partial relaxation. 92–94 – successive stages of relaxation, 95–96 – "complete" concentration 97 – partial relaxation, 98–99 – "complete" contraction to a flattened sphere shape



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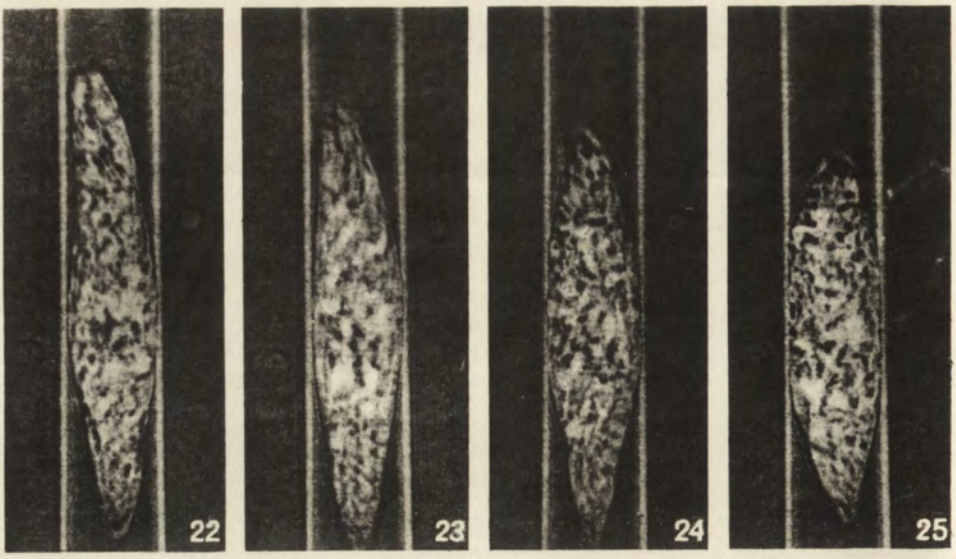

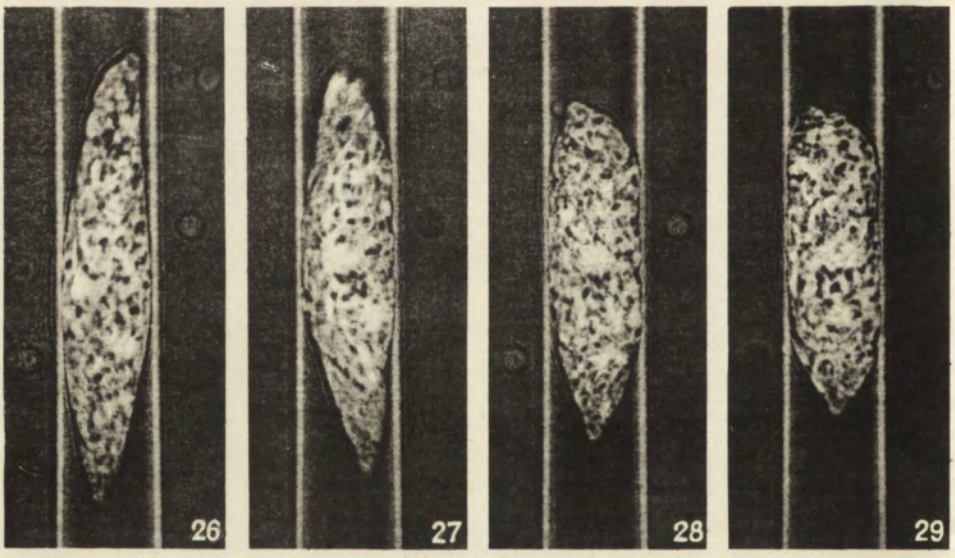

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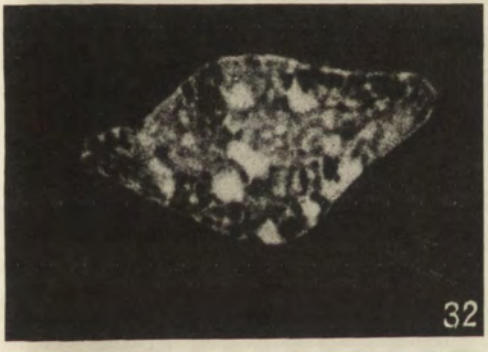

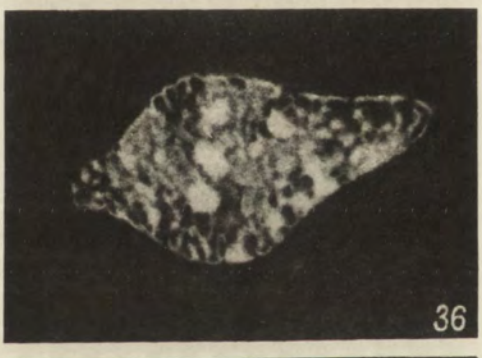

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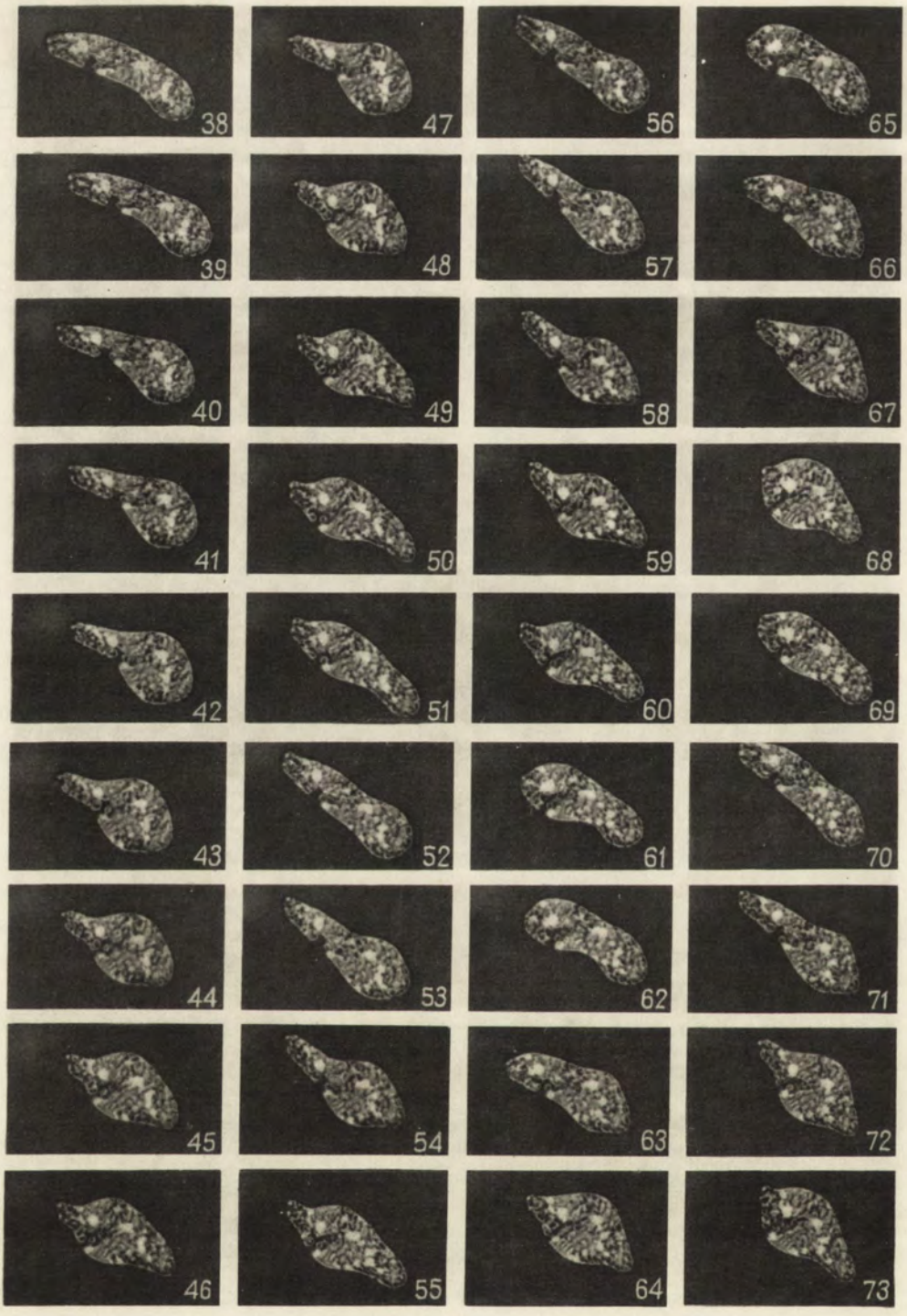

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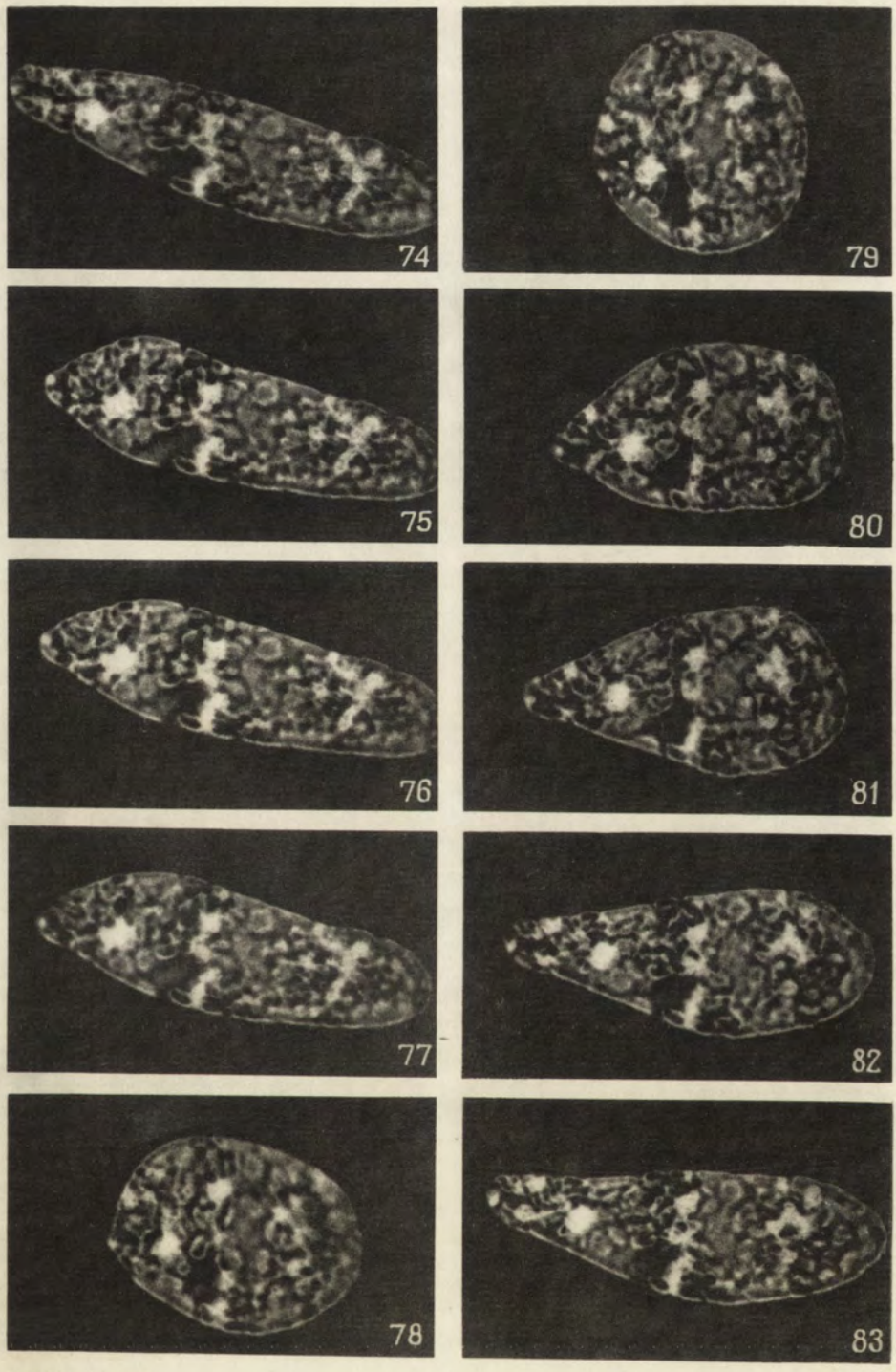

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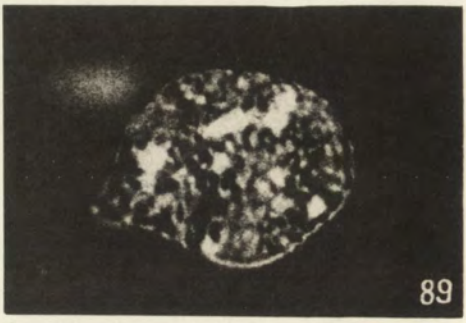

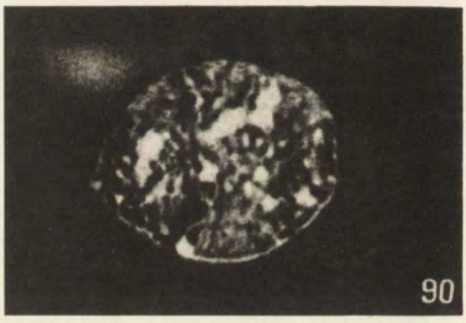

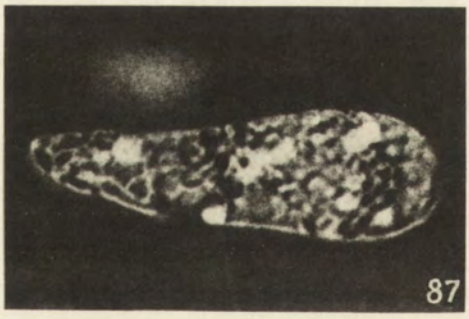

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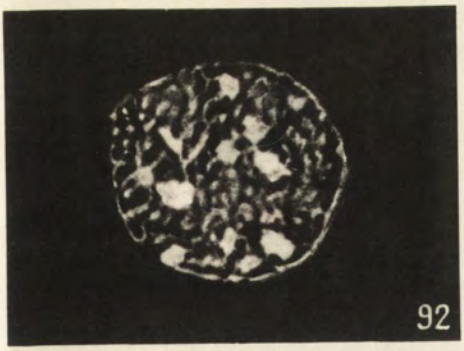

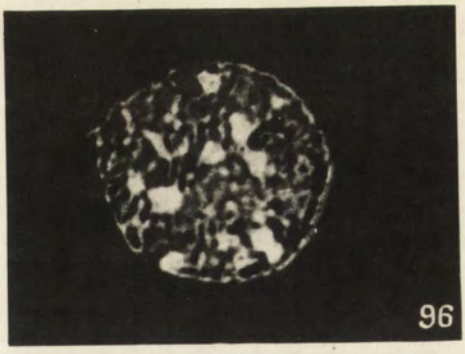

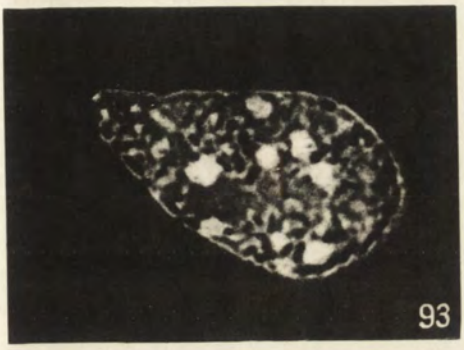

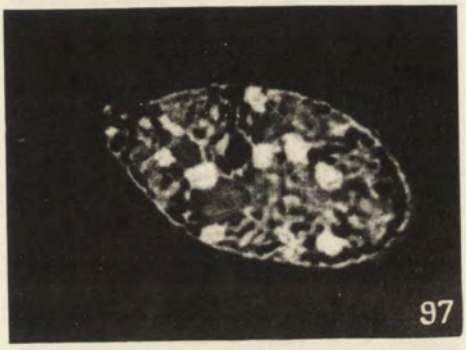

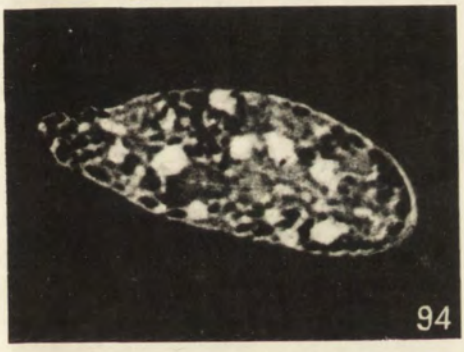

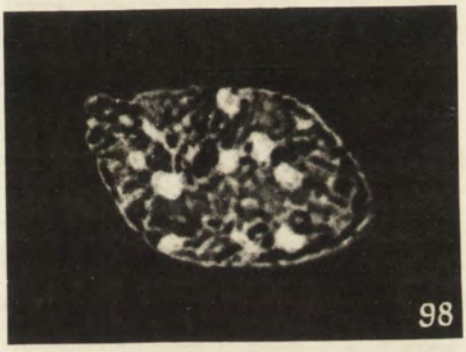

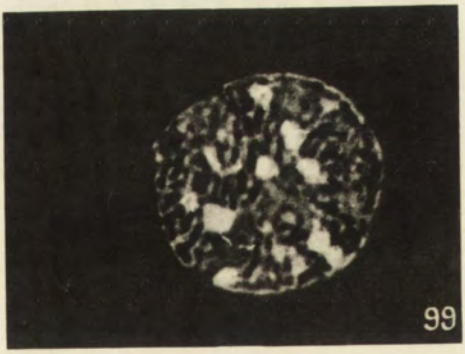

E. Mikołajczyk auctor phot.

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Bodo DIEHN

The receptor/effector system of phototaxis in *Euglena*

This contribution is not concerned with a motile response per se, but rather with the manner in which a motile response is effected in a unicellular organism as a consequence of external stimulation. Since the phenomenon that I will be discussing is phototaxis, the stimulus is unilateral illumination, and the response is orientation with respect to the direction of the light source and subsequent oriented movement of the organism. As will be shown, in *Euglena* phototaxis is a composite response that can be derived from a more basic type of light induced reaction of motile organisms for which the expression "photophobic reaction" (formerly and not quite correctly "phobo-phototaxis") has been coined (Diehn 1969). This is, as the name implies, a nondirectional shock response which can occur either upon a sudden decrease in light intensity (positive photophobic response) or upon an increase (negative photophobic response).

Euglena gracilis is a green alga of prolate ellipsoid shape, usually about 70 to 90 μ long and 15 to 20 μ in diameter (Fig. 1) (Leedale 1967). For the purpose of this discussion, we need concern ourselves only with those organelles of *Euglena* that are directly involved in the receptor/effector chain of light-induced locomotory responses. As the basis of our treatment of the subject, we will divide this chain into four distinct functional units:

(1) The photoreceptor and ancillary organelles.

(2) A signal processing device which converts the information received from the photoreceptor into the input signal for the effector.

(3) The locomotory apparatus.

(4) A device capable of transferring information from (1) to (2) and from (2) to (3).

Our past work has been mainly involved with the nature of the photoreceptor and the mechanism of phototaxis proper. In this paper, I will report on that aspect of the problem, as well as on some recent theoretical work concerning the signal processing that must occur between the receptor and the effector.

The effector (functional unit 3) is, of course, the locomotory flagellum which emerges at the anterior end of the cell. This flagellum usually assumes a trailing

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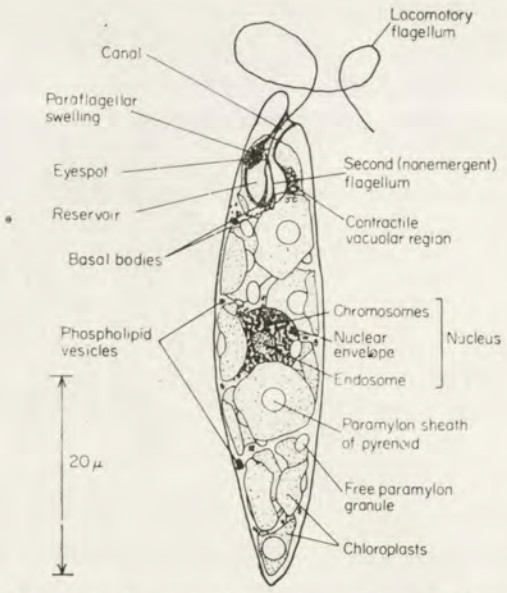

Fig. 1. Schematic drawing of *Euglena gracilis*, after Leedale 1967

configuration during straight line forward motion. At the base of the flagellum near the transition region from canal to reservoir, there is a lateral swelling of about 0.3 μ^2 area which exhibits a patterned structure under the electron microscope (Leedale 1967) and is enclosed within the flagellar membrane. Opposite this "paraflagellar swelling", ensheathing the neck of the reservoir, lies an orange-red pigment spot of about 4 μ^2 cross section. This organelle has been called the "eyespot", but as evidence accumulates that it is not the actual photoreceptor of *Euglena*, the term "stigma" is becoming more generally accepted. The evidence supporting the view that the photoreceptor is contained within the paraflagellar swelling has been summarized elsewhere (Diehn 1970).

Having the photoreceptor attached to the base of the flagellum allows the very attractive hypothesis that the transmission of the phototactic stimulus to the flagellum may be accomplished via the membrane which encloses both the paraflagellar swelling and the flagellum. As in the avoidance response of *Paramecium* (Naitoh and Eckert 1969), a depolarization or hyperpolarization wave might be propagated in the flagellum membrane as the result of stimulation, thus assigning function (4), as listed above, to this structure. We have shown through measurements of action spectra (Diehn 1970) and by indirect chemical evidence (Diehn and Kint 1970) that in *Euglena* the photoreceptor pigment is most probably a flavin derivative. Since photoconductivity has been demonstrated in flavin/phenol charge transfer complexes (Ray et al. 1965), an electrical signal which is related to the light intensity could be generated in such a system if the photopigment were incorporated into

a membrane across which a potential is normally maintained (Jahn 1963). Photoconduction would in this case lead to depolarization of the membrane. Electrophysiological experiments are now underway in our laboratory to test these hypotheses.

When it became apparent that the stigma could not be the photoreceptor proper, we investigated anew Mast's proposal, made 60 years ago (Mast 1911), that the stigma might mediate positive phototaxis, i.e., orientation and movement toward a light source, by serving as a shading device for the photoreceptor. If the stigma's shadow fell on the photoreceptor, the result of the suddenly reduced illumination should be a photophobic response. Our microscopic observations revealed that in *Euglena*, the positive photophobic response appears as a cessation of forward movement, followed immediately by rotation around a lateral axis toward the side which bears the stigma (the "dorsal" side). In the negative photophobic response, occurring upon an increase of light intensity above a critical level, the direction of rotation is reversed. We found that the photophobic response would cease immediately upon restoration of the previous level of illumination. This behaviour, coupled with the fact that the cell rotates slowly around its longitudial axis because of the torque resulting from the helical beat of the flagellum, allows us to explain how positive phototaxis can result from periodic shading of the photoreceptor by the stigma.

In an organism that moves in a straight line path at right angles to the direction of the light (i.e., that has to perform a 90° turn for phototactic orientation), the stigma will shade the photoreceptor once every rotation. At the instant of shading, a positive photophobic response will commence. It will persist until the shadow of the stigma no longer falls upon the photoreceptor. A study of the geometry of the anterior end of *Euglena* indicates that shading would occur for one-third of each turn. Since a positive photophobic response results in a turn toward the stigma, which at the instant of shading was facing the light source, the net result is a fractional turn toward the light. This process is repeated until further shading is geometrically impossible, i.e., until the organism is oriented directly toward the light. Independent support for this hypothesis has been obtained by phototaxis experiments with pulsed actinic light (Diehn 1969).

An entirely analogous mechanism would explain negative phototaxis: Upon high intensity illumination, *Euglena* experiences a negatively photophobic response which continues until the photoreceptor is permanently shaded. If permanent shading were indeed required, the shading function could not be performed by the stigma, though the posterior end of the cell could serve this purpose.

Nothing is at the present known about the hypothetical information-processing unit in our proposed receptor/effector chain. However, an evaluation of Table 1, which summarizes in condensed form the qualitative results of our microscopic observations (Diehn 1969), will make clear why I am suggesting that a signal-conditioning step is interposed between photoreceptor and flagellum:

Table 1

Photophobic responses of *Euglena* as function of light intensity and preadaptation

		Intensity of white light erg/cm²sec		
		8×10^5	2×10^5	5×10^3
Light–adapted	light off	no immediate shock, slight delayed shock	50% shock	shock
	light on after 1.0 sec dark	shock	stops shock shocks previously unshocked cells	stops shock
Dark–adapted	light on	shock	50% shock	no shock
	light off after 1.0 sec illumination	stops shock, delayed 2nd shock	stops shock shocks previously unshocked cells	shock

(a) For any individual cell adapted to a particular light intensity, an immediate photophobic reaction occurs only when the direction of illumination change is away from an "adaption level" which under normal conditions is near 2×10^5 erg/cm² sec. This is evident in the "5×10^3" column. We have, as expected, observed in the analogous case at high intensities that cells adapted to 6×10^5 erg/cm² sec will exhibit a negative photophobic response upon an increase of the light intensity to 8×10^5 erg/cm² sec but will not exhibit a positive photophobic response when the illumination is lowered to 4×10^5 erg/cm² sec. On the assumption that at the output of the photoreceptor appears an electrical signal which is a function of the incident light intensity, the signal processor must be capable of comparing the magnitude of the photoreceptor signal with that of an internal reference level. The processor, furthermore, determines the direction of illumination change, and then generates (or suppresses) a command to the flagellum which causes the latter to alter the direction of its beat toward either the dorsal or the ventral side.

(b) The responses are not "symmetrical" for high and low light intensities. A cell adapted to a low intensity will exhibit an immediate negative photophobic response when exposed to an intensity above 2×10^5 erg/cm² sec, while a cell adapted to the high intensity, will upon darkening show only a delayed and attenuated positive photophobic response. Moreover, both the positive and negative photophobic responses are interdependent. The positive response cannot occur immediately after the negative one ("8×10^5" column) nor is a direct transition from positive to negative photophobic response possible (Diehn 1969).

(c) The receptor/effector chain of a dark-adapted organism can be "reset" for the positive photophobic response by a single light pulse, and vice versa.

On the basis of the above observations, and having available in our laboratory a marvelous toy called the PDP-8 computer[1], we have attempted to answer the question of what kind of processing the raw signal from the photoreceptor might have

Table 2

Focal computer program for simulation of information processing in the phototactic receptor/ /effector chain of *Euglena*

```
C-RK MODV 4-3070

01.05 TYPE !!,"                    PHOTOPHOBIC RESPONSES OF EUGLENA",!!!
01.09 ASK "ADAPTATION LEVEL (LOG I)"AL,!
01.10 ASK "CROSS INHIBITION TIME CONSTANT (SEC)"CK,!
01.11 ASK "EFFECTOR THRESHOLD"TE,!,"EFFECTOR TIME CONSTANT (SEC)"EK",!!
01.12 ASK "LOG STARTING ILLUMINATION"SI,!,"DURATION (SEC)"DS,!,
01.13 ASK "ILLUMINATION CHANGE TO (LOG)"IC,!,"DURATION (SEC)"CD,!
01.14 ASK "LOG FINAL ILLUMINATION"LF,!,"DURATION (SEC)"DF,!!
01.15 ASK "PLOT FROM (SEC)"PF,!,"TO (SEC)"TO,!
01.20 TYPE !!!,"  SEC    LOG I    RESPONSE      RPS",!!!
01.22 TYPE "   0.0   ",%3.02,AL,"     ADAPTED",!
01.30 S ES=0; S I=SI; S EC=0; S EI=0; S EP=0; S TK=EK; S M=.5; S L=.5
01.32 TYPE !,"        ",%3.02,SI,#; FOR N=1,2*DS; DO 2
01.35 TYPE !,"        ",%3.02,IC,#; IF (<SI-AL>*<IC-AL>)1.36,1.36,1.45
01.36 IF (SI-AL)1.40,1.40,1.38
01.38 DO 1.42; SET IP=AL; SET EP=TE+.01; GOTO 1.46
01.40 SET IP=SI; IF (FABS<EC>-TE)1.44,1.44,1.42
01.42 SET EI=ES; S EP=EC; S ES=0; S EC=0; S TK=CK
01.43 GOTO 1.46
01.44 SET ES=0; S EC=0
01.45 SET TK=EK; S EI=0; S EP=0
01.46 IF (FITR<CD+.5>)1.47,1.47,1.48
01.47 SET DC=.5; S M=CD; S L=CD; GOTO 1.49
01.48 SET DC=CD
01.49 SET I=IC; FOR N=(2*DS+1),(2*<DS+DC>); DO 2
01.50 TYPE !,"        ",%3.02,LF,#; IF (<IC-AL>*<LF-AL>)1.52,1.52,1.60
01.52 IF (IC-AL)1.55,1.55,1.54
01.54 DO 1.42; SET IP=AL; SET EP=TE+.01; GOTO 1.61
01.55 SET IP=IC; IF (FABS<EC>-TE)1.59,1.59,1.57
01.57 IF (FABS<EP>-TE)1.58,1.58,1.59
01.58 DO 1.42; GOTO 1.61
01.59 DO 1.44
01.60 DO 1.45
01.61 SET M=.5; IF (FITR<CD+.5>)1.62,1.62,1.65
01.62 SET L=CD
01.65 SET I=LF; FOR N=(2*<DS+DC>+1),(2*<DS+DC+DF>); DO 2
01.99 TYPE !!; QUIT

02.04 SET ES=ES+EC; SET EI=EI+EP
02.10 IF (FABS<EP>-TE)2.30,2.30,2.14
02.14 IF (N-2*PF)2.20,2.15,2.15
02.15 IF (2*TO-N+FITR<1.24-L>)2.20,2.16,2.16
02.16 DO 2.20; DO 2.30; DO 2.39; DO 2.65
02.17 TYPE " /",%2.01,10*FABS<EP>," )",!; GOTO 2.95
02.20 SET EP=(IP-AL-EI)*(1-FEXP<-M/TK>)
02.30 SET EC=(I-AL-ES)*(1-FEXP<-M/TK>)
02.35 IF (N-2*PF)2.96,2.37,2.37
02.37 IF (2*TO-N+FITR<1.24-L>)2.96,2.39,2.39
02.39 TYPE %3.01,.5*(N-1+FITR<.75+L>),"                "
02.40 IF (FABS<EC>-TE)2.90,2.90,2.45
02.45 IF (ES*EC)2.60,2.70,2.70
02.60 DO 2.65; TYPE " )",!; GOTO 2.95
02.65 TYPE "INHIBITED  (",%2.01,10*FABS(EC); GOTO 2.96
02.70 IF (I-AL)2.74,2.90,2.80
02.74 TYPE "POSITIVE    ",%2.01,10*FABS(EC),!; GOTO 2.95
02.80 TYPE "NEGATIVE    ",%2.01,10*FABS(EC),!; GOTO 2.95
02.90 TYPE "ADAPTED",!
02.95 C
02.96 C
*
```

Table 3

Computer printout of simulated response of *Euglena* to changes in illumination level, as in upper half of 8×10^5 column of Table 1

```
GO

   PHOTOPHOBIC RESPONSES OF EUGLENA

ADAPTATION LEVEL (LOG I):5.3
CROSS INHIBITION TIME CONSTANT (SEC):.5
EFFECTOR THRESHOLD:.05
EFFECTOR TIME CONSTANT (SEC):2

LOG STARTING ILLUMINATION:5.9
DURATION (SEC):3
ILLUMINATION CHANGE TO (LOG):2
DURATION (SEC):3
LOG FINAL ILLUMINATION:5.9
DURATION (SEC):3

PLOT FROM (SEC):.5
TO (SEC):9


  SEC    LOG I    RESPONSE      RPS

  0.0    5.30     ADAPTED

  0.5    5.90     NEGATIVE      1.3
  1.0             NEGATIVE      1.0
  1.5             NEGATIVE      0.8
  2.0             NEGATIVE      0.6
  2.5             ADAPTED
  3.0             ADAPTED

  3.5    2.00     INHIBITED   ( 21 / 3.1 )
  4.0             INHIBITED   ( 7.7 / 1.1 )
  4.5             INHIBITED   ( 2.8 / 0.4 )
  5.0             POSITIVE      1.0
  5.5             ADAPTED
  6.0             ADAPTED

  6.5    5.90     NEGATIVE      1.3
  7.0             NEGATIVE      1.0
  7.5             NEGATIVE      0.8
  8.0             NEGATIVE      0.6
  8.5             ADAPTED
  9.0             ADAPTED

*
```

to undergo for the observed response of the flagellar system to occur. Table 2 shows in the Focal computer language [1], a simple series of logical and mathematical operations which will accomplish the necessary data processing. This computer program simulates the following conditions and operations:

[1] Digital Equipment Corp., Maynard, Mass.

(1) At any light intensity, an electrical potential is generated by the photoreceptor which is proportional to the logarithm of the light intensity, and appears instantaneously at the input of the processing network.

(2) An internal reference potential corresponds to the illumination level to which the organism is naturally adapted.

(3) Two storage devices (e.g., membrane capacitances), can be charged (or discharged, as the case may be), toward the receptor or reference potential level. If the charge rate exceeds a threshold value, the effector is activated; otherwise the system is considered adapted. The second of the storage devices is utilized only when a change in light intensity is imposed upon the system while it has not yet adapted to the previous illumination, i.e., when the first capacitance is incompletely charged or discharged. When both storage devices are utilized, charge and discharge occur at an accelerated rate, and the signal activating the effector is suppressed.

Thus programmed. our computer simulates the photophobic responses of *Euglena* quite accurately. Table 3, for instance, is the computer printout of a simulation of the "Light-Adapted" experiment at 8×10^5 erg cm^2 sec in Table 1. In several non-trivial instances, we have, in fact, done computer simulations of previously not attempted experiments, and subsequently observed the behavior which had been predicted by our model. At present, the agreement between observed and simulated behavior is only qualitative. As one starts to quantitatively study the duration and strength of the photophobic responses, certain inadequacies of our model become evident. We are, in fact, at present seriously considering the possibility that *Euglena* may have two photoreceptors, one for high light intensities and one for low intensities. This is based on the difficulties we are experiencing in our attempts to have a model with only one photoreceptor account quantitatively for all observed data. Nevertheless, we seem to have a working hypothesis which can be refined further as more experimental evidence accumulates.

I should point out that a digital computer is not the only nor possibly the best device for simulating phototactic behavior. Instead of using a digital approach, one could use our model to construct with very few parts an electronic analog of a *Euglena* cell which would behave in a perfectly life-like manner with respect to motile behavior upon stimulation.

Acknowledgement

Some of the work described herein was supported by Grant No. GB-18701 from the U.S. National Science Foundation.

Summary

Studies are described concerning the mechanism by which the unicellular green alga *Euglena* orients itself with respect to an incident beam of light. The photoreceptor pigment has been tentatively identified as a flavin derivative in a hydrophobic environment. On the assumption

that the photoreceptor signal as well as the control mechanism for the flagellar beat are electrical in nature, a sequence of signal-processing steps between receptor and effector has been postulated and incorporated in a Focal computer program. This program accurately simulates the motile behavior of *Euglena* upon stimulation with light.

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Positive chemotactic reactions of *Amoeba proteus* to general anaesthetics

For the last few decades the studies of amoeboid movements have concentrated on the processes securing generation of the forces responsible for intracellular streamings of protoplasm. In this field great progress has been achieved. It has been demonstrated that the systems of contractile proteins similar to those found in specialized tissue of muscles are present in various types of motile cells including *Protozoa*. Basic mechanochemical processes of conversion of chemical into kinetic energy also appeared to be similar in all living cells, though the structural arrangement of contractile materials can vary, depending on the organisation of particular cells (Sinard-Duquesne and Couillard 1962, Wohlfarth-Botterman 1964, Kamiya 1966, Allen 1968, Hatano and Tazawa 1968). There are, however, great gaps in our understanding of cellular locomotion. These concern above all the mechanisms which control the operation of cellular contractile systems. Especially in the case of amoeboid movements, in spite of the constant study of the phenomenon, surprisingly little is known of how locomoting amoeba keeps the spatial polarization of its protoplasmic transformations and contractile reactions. What is more, there are fairly sharp differences in opinion concerning the localization of the contractile processes providing forces for the intracellular axial flow of the endoplasm into extending pseudopodia.

Suggestions have been made that the contractile processes in cells moving in the amoeboid manner can be influenced or controlled by properties and functions of the cell membrane, but direct evidence concerning large amoebae was lacking. Bingley et al. (1962) found that extracts of *Hydra* can induce pseudopodia formation in *Amoeba proteus*. Though these authors came to the conclusion that the active material interacts with the amoeba membrane they did not explain definitely what is the mechanism of this interaction and how the changes in the cell membrane properties can influence the processes of protoplasmic contraction. Their suggestion that the electric gradients along the amoeba cytoplasm may be responsible for the polarization of cytoplasmic streamings (Bingley and Thompson 1962) appears premature in the light of more recent experimental results (Batueva 1964). Also

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

the chemical nature of the active substance obtained from *Hydra* remains obscure (cf. Jeon and Bell 1965).

Recently it has been demonstrated fairly conclusively that in slime mould plasmodia, the activities of which resemble the movements of large amoebae, the streamings of protoplasm and the sol-gel reactions are controlled by the activities of plasma membranes. When the activities of the plasma membrane were locally modified by an anaesthetic, benzamide, the ectoplasmic contractions were locally weakened, the sol-gel cytoplasmic reactions affected, and a long-lasting inflow of the protoplasm into the treated area of plasmodium occurred. These phenomena were reversible and appeared to be associated with characteristic modifications of electric activity of the plasmodia (Korohoda et al. 1969, 1970). These results raise the question whether in an amoeba the activities of the cell membrane control cellular movements as well. To examine this hypothesis the simple experiments described below were carried out.

So far the study of chemotactic reactions in amoeboid cells (amoebae, leucocytes, etc.) were based on search for active substances by examination of various extracts from biological materials. However, such extracts contain a very complex mixture of various chemical substances and the isolation and identification of the active one is very difficult. Therefore we started experiments with general anaesthetics which are well known to influence the cell membrane functions.

Amoeba proteus cultured on Chalkley's medium provided the material for the experiments. Several amoebae were chosen and put in a hanging drop of the medium under a cover glass of the moisture chamber of a Zeiss sliding micromanipulator. The reactions to general anaesthetics of amoebae attached to the glass and actively cocomoting were observed later. The tested substances were placed in a glass micro lapillary, as shown in Fig. 1. The direct filling of the capillary with volatile anaesthe-

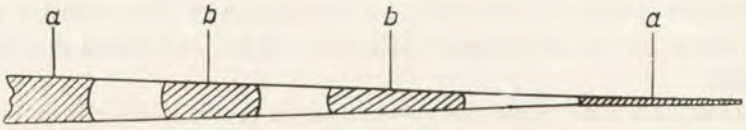

Fig. 1. Microcapillary containing a volatile anaesthetic, a—Chalkle's medium saturated with anaesthetic, b—droplets of anaesthetic

tics appeared inappropriate since later the tested substances leaked out into the medium too rapidly. For the experiments with benzene the diameter of the capillary tip was aprox. 5 μm, for ether and chloroform aprox. 2 μm. The anaesthetics, which saturated a small volume of the medium filling the fore part of the microcapillary, diffused into the medium surrounding the amoebae, securing in it a local gradient of concentration of the tested substance.

It was found that general anaesthetics such as benzene, chloroform, and ether induced the cell response identical with those which Bingley et al. (1962) described for extracts of *Hydra*. When the tip of the capillary containing the anaesthetic was

placed near the cell surface, the amoeba immediately produced pseudopodia directed towards it. If later the capillary was slowly shifted, the amoeba followed the tip in its movements (Pl. I 1–4). The amoebae followed the tip of the capillary for dozens of minutes even if it was shifted in a zigzag way. These reactions were observed whenever the tip of the capillary came close to the amoeba. Only when the cell uroid was exposed to the anaesthetics did its conversion into extending, dominating lobopodium occur somewhat rarely. More often a thin hyaline pseudopodium was produced which was later withdrawn. The cell regions at the tips of advancing pseudopodia appeared most sensitive. The amoebae which followed the capillary for a considerable period of time (up to 70 min) showed no difference in reaction in comparison with the amoebae exposed to anaesthetics for the first time. These reactions of amoebae seem to be directly related to the anaesthetic action of benzene, chloroform and ether upon the cell membrane. Carbon tetrachloride, which also is fat soluble, induced an inflow of the cytoplasm into the exposed amoeba region but later the progressive cell movements were inhibited. An increase in the cell membrane surface area, which probably occurs due to the action of lipid soluble anaesthetics (cf. Goldacre 1952) cannot be responsible by itself for the observed responses of amoebae, which concern not only the production of pseudopodia but also changes in the direction of cellular locomotion and in polarization of the intracellular sol-gel reactions.

The power of the general anaesthetics to induce the production of pseudopodia in *Amoeba proteus* and to modify the polarization of its movements and internal structure points to the conclusion that, as in the plasmodia of slime mould, also in amoebae the activities of the cell membrane control the release of contractile and sol-gel cytoplasmic reactions. In the light of the above results previous reports that local administration of carbon dioxide induced pseudopodia formation (Jacobs 1922) and that an oil drop if attached to the cell membrane stimulated formation of pseudopodia and determined permanently the monopodial movements of amoebae (Dawson and Belkin (1928, 1929)) have become explicable. Both of these agents can be expected to inhibit locally the membrane activities associated with the processes of exchange of ions between the cytoplasm and the medium. These last processes can be assumed fairly safely to initiate a similar chain of events leading to intracellular contractile reactions as occur in muscles. The cell membrane activities which are sensitive to external factors appeared to play an important role in the control of amoeba locomotion. And the statement of J. D. Bernal (1965) that: "Motion in itself in an organism would be of very little value if it was not combined with the corresponding mechanism for sensation" appears to be correct not only when analysing the motile behaviour of multicellular organisms but also for individual cells locomoting in the amoeboid manner.

Acknowledgement

The author expresses his gratitude to Prof. E. J. Ambrose for encouragement and critical reading of the manuscript.

Summary

It was observed that benzene, chloroform and ether when applied locally to *Amoeba proteroteus* induce pseudopodia formation. Amoebae not only produce pseudopodia extending towards the tip of microcapillary filled up with medium saturated with these anaesthetics, but later follow the capillary for dozens of minutes when it is shifted in a zig-zag way. This observation is discussed in terms of the significance of cell membrane functions for control of intracellular contractile and sol-gel cytoplasmic reactions.

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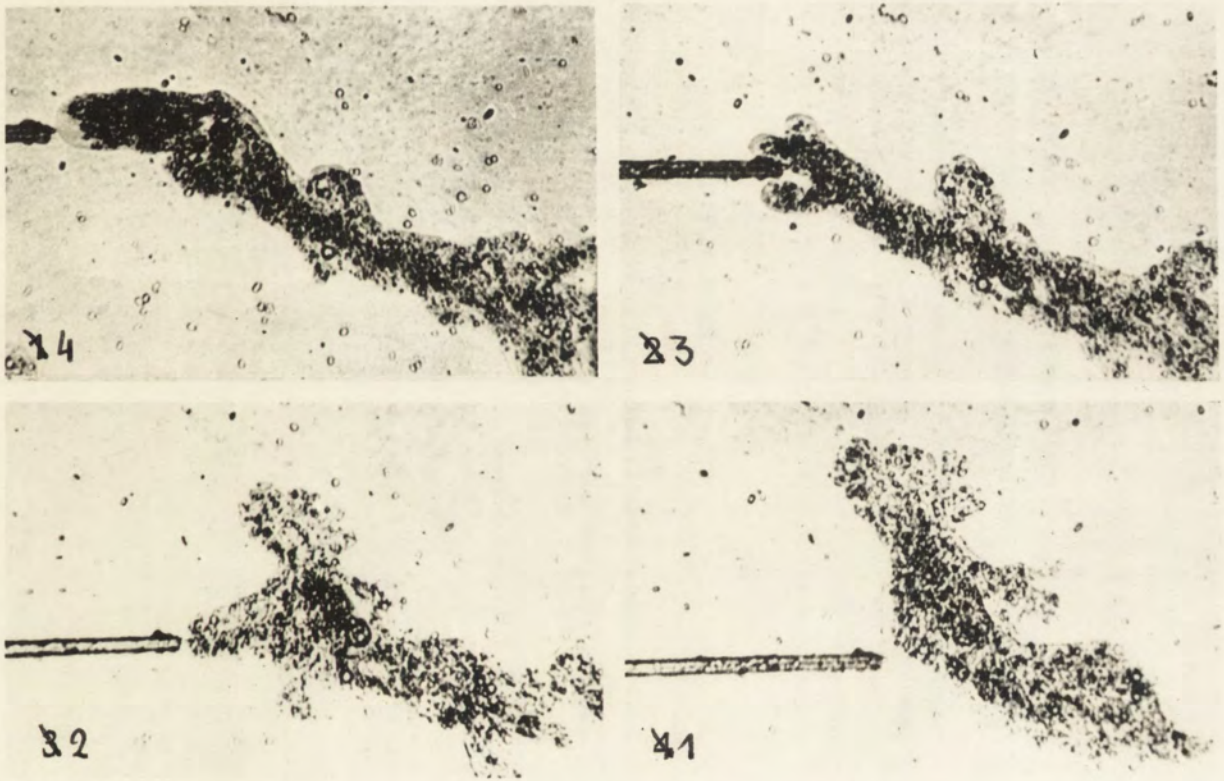
EXPLANATION OF PLATE I

1–4: *Amoeba proteus*, photographed in successive positions, showing pseudopod formation stimulated by proximity of the capillary containing benzene



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The role of elastoosmotic parameters in locomotion of myxomycete plasmodia

It has been suggested by some authors that the superficial gel layer and membrane are the motile organelles in amoeboid type of locomotion.

Of particular interest are some ideas about the role of the plasmagel tension (Mast 1926, Lewis 1942), of the intracellular hydrostatic pressure (Mast 1926, 1931, Stewart 1964) and of the active expansion of the plasmalemma (Bell 1961, 1963) in the locomotion of the cell. If the intracellular pressure is balanced out by opposing elastic forces of the superficial layers (or "physiological membrane") of the cell one may expect that the local changes in the elasticity of these layers may result in the protoplasmic flow and cell locomotion.

If the cell membrane is semipermeable (this is generally accepted for all cells) then in the equilibrium state the positive hydrostatic pressure must be an osmotic one. Even in the case of active contraction of the cell membrane in an isotonic medium the water will flow out from the cell untill the intracellular osmotic pressure caused by the difference in concentration becomes equal to the pressure of the active contraction.

Obviously, if the semipermeability of the membrane is not observed, the contraction will cause a change of volume rather than a change in the intracellular pressure.

When describing the system in the stress-strain terms, the tension state of the cell membrane during active contraction of actomyosin complex does not differ logically from "the passive tension" arising upon the increase of the osmomolarity of the cell solution. In both cases the osmotic pressure would rise and the behaviour of the system in the normal condition and in reological experiments will be determined by the water permeability of the membrane. Therefore in this study the following questions are considered:

(1) What are the osmotic properties of a plasmodium and the experimental volume-osmotic pressure relationships?

(2) What are the theoretical volume-osmotic pressure relationships for an elastic osmotic shell?

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(3) What is the influence of the tension of the plasmodial envelope on the motive force of protoplasmic flow as well as on the dynamics of the intracellular water?

Let's consider a model of the cell with the elastic membrane in the form of the spherical shell. The term "membrane" is used here generally. It means cell envelope, e.g. membrane plus cortical layer and external fibrillar coat or membrane plus cortical layer.

The main parameters of such a shell are the following:

V_0 – the volume of the shell with the fully smoothed membrane without stresses in it.

φn – a number of osmoles of solute.

E and ν – Young's modulus and Poisson's ratio of the membrane.

V_d – "osmotic dead space".

$H=\frac{h}{r_o}$ – the relative thickness of the membrane, where r_o is the radius of the shell having the volume V_o.

Some assumption must be introduced:

(1) The membrane of the shell is semipermeable, being permeable only for water and not permeable for the solute under consideration.

(2) The membrane of the shell is freely deformable and determined by the general principles of the elasticity theories such as the principle of superposition and the generalized Hooke's law.

(3) The membrane does not possess flexural rigidity.

(4) The volume of the membrane does not change during the deformation that is Poisson's ratio is equal to 0.5 (the usual value used for the biological objects).

Let's put such an osmotic shell into a solution having an infinite volume with the C_e concentration. Since the condition for equilibrium of such a system is an equality of chemical potential of solvent, this equilibrium is achieved either by equalizing the outer and inner concentrations (the condition of an absolutely expansible membrane), or a pressure gradient appears (the condition of an absolutely rigid membrane). The living cells are in an intermediate state; their membranes possess some elasticity.

Let the shell volume at the equilibrium be V. Than the inner concentration will be equal to $C_i=\frac{\varphi n}{V-V_d}$ and according to Van't Hoff's law one can write;

$$\Delta p=\frac{RT\varphi n}{V-V_d}-RTC_e: \tag{1}$$

This law is valid for very dilute and ideal solutions that is if the interaction among components of the solution is not taken into consideration. To remove these restrictions for the real solutions it is necessary to introduce the osmotic coefficient φ. In this case the concentration of the solution is expressed in osmoles per kg of water:

$C=\varphi\ m$, where m – is the molar concentration of the solution. The inner osmolar concentration is a function of only the shell volume if we suppose that φn and V_d are the constants. The volume in turn is a function of the concentration. Let the volume V_0 correspond to some outer concentration C_e^0. At this volume (according to the definition) the membrane tension is lacking, that is the pressure $\Delta p=0$, and the inner and outer concentrations are equal:

$$C_o^i=\frac{\varphi n}{V_o-V_d}=C_e^o. \tag{2}$$

When $C_e^0<C_e^0$ (hypotonic range) then $V>V_0$ that is the tension arises and $\Delta p\neq 0$. At $C_e>C_e^0$ (hypertonic range) $V<V_0$, the membrane is folded and considering the third assumption, $\Delta p=0$, i.e. over this range $C_i=C_e$. Taking into account (2) expression (1) may be written:

$$\Delta p=RT\,\frac{V_o-V_d}{V-V_d}\,C_e^o-RTC_e, \tag{3}$$

i.e. the inner concentration may be presented as:

$$C_i=\frac{V_o-V_d}{V-V_d}\times C_e^o. \tag{4}$$

Hydrostatic pressure arising in the shell, caused by the tension of the elastic membrane (second assumption) is related to its physical parameters by the equation:

$$\Delta p=\frac{hE(V-V_o)}{0.9(I-\nu)\times V_o\sqrt[3]{V_o}}. \tag{5}$$

This equation may be deduced from the elasticity theory of the plates and shells, developed by Timoshenko (1940).

Taking into account the forth assumption and the fact that elasticity begins to take part only when the concentration $C_e<C_e^0$, in the mathematical form it may be written:

$$E(C_e)=E\times 1(C_e^o-C_e),$$

where

$$1(C_e^o-C_e)=\begin{matrix}0\\1\end{matrix}\left\{\begin{matrix}\text{at}\quad C_e\geqslant C_e^o\\ \text{at}\quad C_e<C_e^o\end{matrix}\right.$$

equation (5) may be expressed in the form:

$$\Delta p=1.3HE\times 1(C_e^o-C_e)\frac{(V-V_o)}{V_o}. \tag{6}$$

Combining this expression with (3) we will have:

$$RTC_e^o\frac{V_o-V_d}{V-V_d}-1.3HE\times 1(C_e^o-C_e)\frac{V-V_o}{V_o}=RTC_e \tag{7}$$

or in dimensionless terms

$$x=\frac{y}{1+ya\times 1(x-1)-y^2\times a\times 1(x-1)}, \tag{8}$$

where

$$y=\frac{V-V_d}{V_o-V_d}; \quad x=\frac{C_e^o}{C_e}; \quad a=\frac{1.3HE(V_o-V_d)}{RTC_e^o \quad V_o}.$$

This equation relates indirectly the volume of the osmotic shell with a given concentration of the outer medium.

Figure 1 gives the curves of this dependency at the different values of the parameter "*a*".

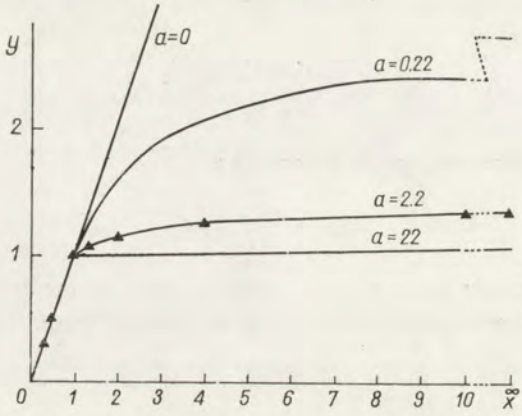

Fig. 1. The dependence of ideal osmotic shell volume on the reverse concentration of external medium. The curves are obtained from the equation (8) at different values of parameter "*a*". The points correspond to experimentally obtained values for plasmodium myxomycete

If for the osmotic shell "*a*" $\neq 0$ then at $x>I(C_e<C_e^0)$ a hydrostatic pressure exists, i. e. the membrane of the shell is under tension. If the value *HE* changes simultaneously throughout the membrane then the shell volume is changing untill a new equilibrium state is achieved. The local change of *HE* causes first of all a change of the shell form and a displacement of the inner solution provided that there is an adhesion between the shell and the substrate. This model can explain the ameboid movement if the value of *HE* periodically changes. At some values of viscosity and frequence of changes in *HE* the pressure gradient does not occur in the shell, i. e. displacement of the solution will take place only during the change of the value *HE*.

Materials and methods

Slime mold *Physarum polycephalum* grown by the method of Camp (1936) was chosen as an object for investigation.

Experiments on the influence of the osmotic pressure of medium on the volume of minute

pieces of plasmodium were carried out by two methods. Optical method: from the plasmodium the minute pieces were cut off, which became spherical or ellipsoidal when the medium was stirred. The diameter of such pieces ranged from 100 to 200 μ. The measurements were carried out using an eyepiece micrometer. The value of division was 6.25 μ, the measuring error was ±5%. Weighing method: to measure the weight of the plasmodium pieces a torsion balance with scale spacing equal to 0.05 mg has been used. The weight of the pieces ranged from 0.6 to 2 mg. Pieces of plasmodia from a control solution were put on filter paper for 2–3 sec for removal of excess water. They were then quickly weighed and transferred to an experimental solution. A set of control weighings in the same solution showed that the error of the method did not exceed 3–5%.

The motive force of the protoplasmic flow was measured using the method of balance pressure (Kamiya 1942). To study the influence of osmotic pressure of the medium on the motive force plasmodial blebs in the both compartments of the double-chamber were treated with sucrose or glucose solution. The influence of anaesthetics on the motive force was studied by applying microdrops on the plasmodial net or on its front parts.

In experiments on the endoplasmic polarization the parts of plasmodial strands, both poor and rich with endoplasm (experimentally), were excised, quickly weighed, and put in water. After 20 min incubation, the weighing was repeated.

Solutions

Before each experiment the plasmodia were incubated in Prescott–James solution (Prescott and Carrier 1964), or in tris-buffer (2 mM, pH 6.9). The effective osmotic pressure of the sucrose solutions was calculated according to the following formulae (Robinson et al. 1942):

$$C_e = 0.0804\, x^2 + 29.24\, x + 1 \quad (\text{mosM/kg } H_2O).$$

where x is the weight of the saccharose in grams of the solvent.

Benzamide was prepared on the phosphate buffer pH 6.9 in concentration 25–50 mM (Korohoda et al., 1969). Hexenalum (40 mM) and urethan (2.5%) was prepared on the Prescott–James solution or on the tris buffer (pH 6.9).

Results and discussion

The demonstration of the hydrostatic pressure inside the plasmodium

The results of experiments on punctures under atmospheric pressure show that throwing out of the liquid endoplasm occurs only in strands which can be morphologically distinquished. These are located in the central part of plasmodial surface culture, almost ideally round in cross-section and have a thinner cortical layer of plasmagel. Their role seems to be connected exclusively with the endoplasm transfer from one part of the plasmodium to another.

It is characteristic that the endoplasm exudation is not observed after the puncture in caudal and frontal regions. Considering that plasmodium has no inner partitions it suggests that the above mentioned zones differ as to inner structure and are likely to be in a gel state. Gel is characterized by swelling pressure which can be detected only by an osmotic method. If one applies to the plasmodia a vibrating needle the zone around its tip becomes liquid thus pointing to the presence of gel and its thixotropy.

Kinetics of plasmodial volume changes in nonelectrolyte solutions

Since the osmotic experiments were carried out by a weight method, considering the weight of pure water to be proportional to its volume, one can write down:

$$y=\frac{V-V_d}{V_o-V_d}=\frac{P-P_d}{P_o-P_d}, \tag{9}$$

where P – is the plasmodial weight corresponding to the concentration C_e,

P_o – is plasmodial weight corresponding to C_e^0, i.e. volume V_o,

P_d – is the weight of insolubilizing volume corresponding to infinite external concentration.

Figure 2 shows the typical curves of the change of relative plasmodial weight/the

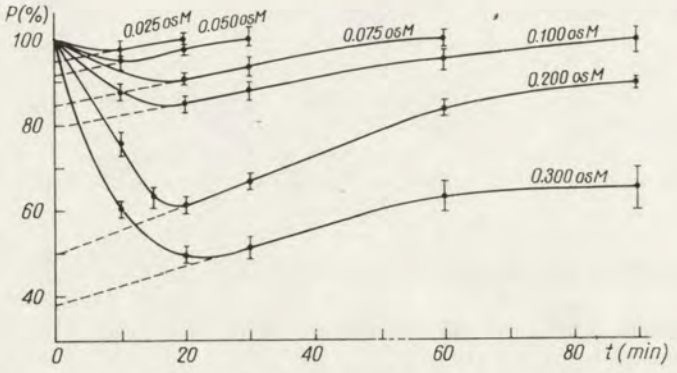

Fig. 2. The kinetics of the change of relative plasmodial weight in the different concentrations of the sucrose solution

weight of a plasmodium in Prescott-James solution equal to the weight in water in the limits of the method error, ($C_e=0$) in nonelectrolyte solutions. The character of the change of plasmodial weight in sucrose solutions resembles the kinetics in so-called "relaxation" experiments (Laidler and Shuler 1949) with substances penetrating the cell membrane: at first the weight drop occurs, thereupon in 20–30 min there is an increase of weight. In solutions up to 100 mosM/kg of water one can observe the restoration of the initial weight in 40–60 min to the control level.

The expression (8) being strictly thermodynamic, i.e. dealing only with steady states, the question arises if such data can be used for experimental checking.

A similar situation appears when measuring the osmotic pressure of solutions in the presence of a "partially permeable" membrane. The theoretical approach to this problem on the basis of thermodynamics of irreversible processes shows that the significant experimental value is "apparent" osmotic pressure at the stationary state during which the liquid flow due to the chemical potential gradient is balanced out by the flow resulting from the hydrostatic pressure (Morawetz 1967).

This stationary pressure is related to the osmotic pressure at the state of thermodynamic equilibrium (π_{ideal}) in the following way: $\pi_{apparent}/\pi^{i}_{deal}=\sigma$, where σ is the coefficient of selectivity characterizing the membrane.

Consider the volume as a single-valued function of medium concentration, then such a significant experimental value is the minimal volume (proportional to the weight). If the coefficient of membrane selectivity were known, it would be possible to determine the equilibrium value of volume for the ideal case when the membrane is impermeable to a given substance.

The process of volume restoration can be connected, at least, with three mechanisms: sucrose diffusion inside, pinocytotic absorption of solution and change of the osmotically active concentration of protoplasm at the cost of internal processes.

The curves with glucose and raffinose (molecular weights differ about three times) coincide with sucrose curve of the same concentration (Fig. 3) within the limits of the method error ($\pm 3\%$). It suggests that the increase in the internal osmolal concentration is not connected with nonelectrolyte diffusion through the membrane. As glucose enters the plasmodium (qualitative reaction with Felling reagent), one can assume that weight restoration is connected with nonelectrolyte penetration through another process, apparently, pinocytosis.

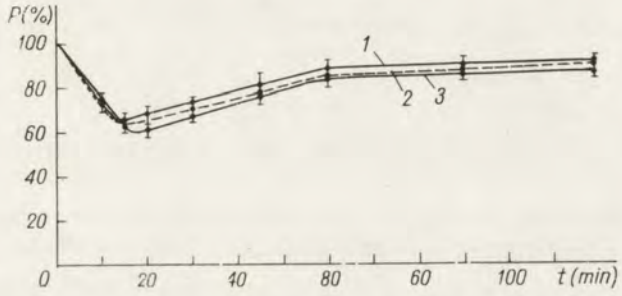

Fig. 3. The kinetics of the change of relative plasmodial weight in solutions of nonelectrolytes in concentration 200 mosM/kg H_2. 1—glucose, 2—rafinose and 3—sucrose

Volume restoration is linear, that is why the extrapolation to the time zero gives the equilibrium volume values. Thus, the volume dependence on the medium concentration constructed according to these values can be compared with theoretical relationships obtained from the expression (8).

Comparison of theoretical relationships with the experimental one obtained for myxomycete plasmodia

Figure 4 represents the experimental curve. On the ordinates the relative plasmodium weight is plotted obtained by extrapolation of kinetic curves to the time zero (see Fig. 2).

Extrapolation of the linear part of this curve to the point of the infinite external concentration gives the relative weight of unsolubilizing volume equal to 0.2. Considering that protein concentration in plasmodium constitutes 10% (Andersen 1962) and that the protein density is equal to 1.35 g/cm^3, it is possible to find the relative unsolubilizing volume Vd/V to be equal to 17.4%. In recent years high values of unsolubilizing volume (almost 35% for red blood cells) obtained with the help of extrapolation and the large percentage of bonded water were revised (Dick 1959, Savits et al. 1964, Gary-Bobo and Solomon 1967).

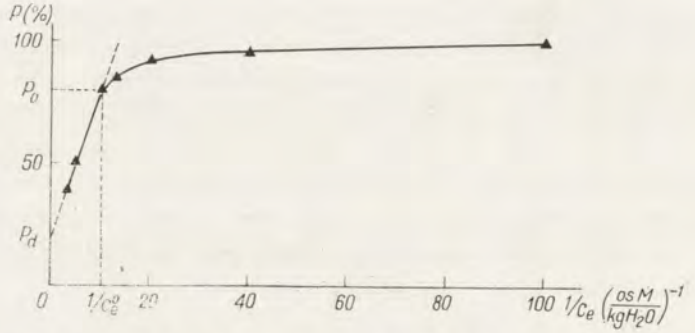

Fig. 4. The dependence of relative plasmodial weight on the reverse concentration of externa medium

Dick (1959) came to the conclusion that under the osmotic stress the condition $\varphi n = \text{const.}$ is disturbed. That was confirmed experimentally on erythrocytes (Gary-Bobo and Solomon 1967).

In our experimental curve this effect was eliminated by the extrapolation of the kinetic curves to the time zero. Possible error, therefore, can be caused by only two reasons: methodical one connected with the difficulty of considering a water film on the plasmodial surface and the possible nonlinearity of the process of volume restoration during the first minutes of incubation in nonelectrolyte solutions.

The curve of dependence at concentrations lower than 100 mosM/kg of water has sharply defined nonlinear character. This concentration (C_e^0) corresponds to the volume of a plasmodium with completely unfolded membrane and to the beginning of elastic behaviour of the membrane, i.e. to the appearance of intracellular hydrostatic pressure. From the graph it is seen that $P_o = 0.8$.

Experimental points computed in dimensionless values according to the expression (9) using $P_o = 0.8$; $P_d = 0.2$ and $C_e^o = 0.1$ osM/kg water in the limits of the method error at all concentrations are applied to the theoretical curve constructed at "a" = = 2.2 (Fig. 1).

It will be recalled that $a = \dfrac{1.3\, HE\,(V_o - V_d)}{RTC_e^o \qquad V_o}$, so it is possible to determine the value HE for a plasmodium. It is equal to 5.4×10^6 dyne/cm^2. Considering that

the elasticity of a plasmodium is concentrated completely in the surface layer of the relative thickness $H=0.1$ we get $E=5.4\times10^7$ dyne/cm^2. The internal concentration of plasmodium, when it is in pure water, can be calculated according to expression (4). It is equal to 70 mosM/kg water. This value is in good agreement with the data obtained by Andersen (1962), who showed that myxomycete contains about 30.0 mekv/kg potassium and 8.0 mekv/kg sodium. This concentration is followed by hydrostatic pressure $P=1.7\times10^6$ dyne/cm^2 (determined according to (7). The reality of such value is confirmed by literature data.

After Løvtrup and Pigon (1951) the osmotical active concentration of amoeba accounts to 80 mosM/kg of water. Bozler (1965) has obtained the experimental relationship between the osmotic activity of the external medium and the weight of smooth muscle. He considers the nonlinearity of this relationship to be a result of restricted swelling of the calcium bonded muscle gel. The calculated swelling pressure was 1.6 atm.

By the puncture method the inner pressure in Fundulus eggs was measured in undiluted sea water (Kao and Chambers 1954). After the puncturing it increased appreciably and reached 1.2 atm. Doerner (1967) proposed a "transposing volume" method for the analysis of the pressures and stresses in cells. The calculated intracellular pressure for *Amoeba proteus* was 1.6 atm.

In osmotic experiments on sea-urhin eggs Mela (1967) has shown that on definite dilution of sea water further increase in medium hypotonicity results in a nonlinear volume-osmotic pressure relationship. According to Mela value of elastic modulus for membrane of the eggs is 1.0×10^7 d/cm^2 (it should be noted, that Mela has not proved that $\varphi n=\text{const}$).

Corresponding values of the modulus elasticity were obtained for the fibrous sheet of tail of bull spermatozoa (Rikmenspoel 1965), for the extracted muscle fiber (Bozler 1964), and the membrane erythrocyte (Katchalsky et al. 1960, Rand and Burton 1963).

For the myxomycete plasmodium Norris (1940) has obtained the elasticity modulus values of about 10^5 dyne/cm^2. It differs from the presented value by two orders of magnitude. This difference seems to be explained by the fact that in his experiments the force relates to the cross-section of strand. On the swelling of the shell the tension relates to the membrane thickness, therefore the elasticity is calculated only for the material of the envelope. Besides, he carried out experiments on strands being already stretched by the inner pressure.

The influence of the degree of swelling on the motive force (MF) of a plasmodium

Inasmuch the osmotic behaviour of plasmodium is satisfactorily described by the considered model, it can be expected that there exists a causal relationship between the swelling pressure and MF of the protoplasm measured with the help of

balance pressure. While measuring the MF when the intracellular pressure is artificially increased or decreased a periodic non-equilibrium state will arise and the influx and outflux of water must occur. Therefore the magnitude MF will depend not only on the contraction force, but on the water influx (or outflux) rate determined by the filtration coefficient (as calculated from kinetic curves $P_w = 2.6 \times 10^{-9}$ cm/sec cm H_2O).

The degree of swelling (intracellular pressure) was regulated either by the change in the tonicity of the medium (since plasmodium lives in fresh water, it is possible to study only hypertonic solutions) or by the degree of filling the strands with the endoplasm from adjacent regions of plasmodium.

In Fig. 5 the change in MF under the action of glucose solution with a concen-

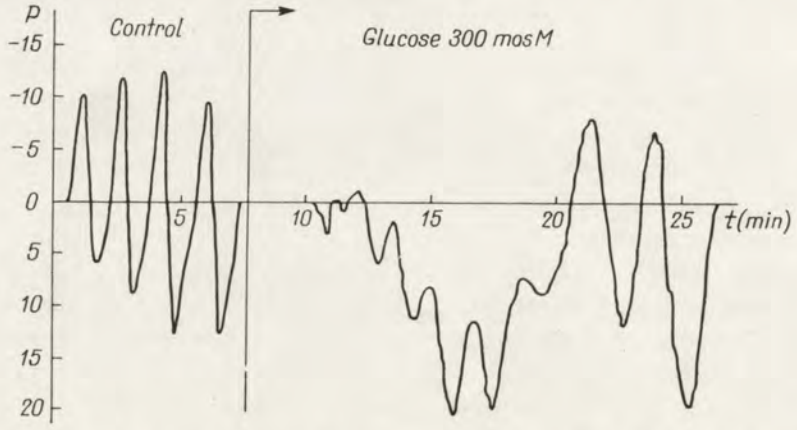

Fig. 5. The influence of glucose solution in concentration 300 mosM/kg H_2O on the motive force

tration of 300 mosM/kg H_2O is shown. A marked decrease in the amplitude of oscillations of the MF occurs only during the first 6–10 min after the beginning of incubation. Then, amplitude increases may be due to the penetration of glucose into the cell (see the kinetics of volume restoration in solutions of nonelectrolytes, Fig. 3) and an increase of the intracellular pressure.

The main requirement for obtaining accurate results on the action of nonelectrolytes is small dimensions of plasmodial strands. When long strands are used, the disturbance of rhythm and synchronous action of plasmodial activity centres occurs which hinders the application of counterpressure. This reaction may be due to unequal permeability of different regions of the plasmodium membrane to water. Morphological observations showed that in the solutions of sucrose protoplasmatic strands acquire a specific bead-shaped form, that is the protoplasm collects to dense clusters located along strands at regular intervals. On prolonged incubation or when being transferred into water these clusters resolve. This observation assumes arti-

culated structure of plasmodial strands with different functional states of the membrane.

When sucking off the endoplasm or filling the protoplasmic strands with the endoplasm from adjacent parts of plasmodium which are subsequently cut off (instantaneous formation of the membrane on the surface of the cut isolates the system from the medium) one can change the degree of the envelope tension. The amplitude of MF oscillations is directly proportional to amounts of the endoplasm. However, in 10–20 min it returns to the control value (Kamiya 1968).

On the shift of the endoplasm in response to the artificial pressure gradient (30 cm of water in Fig. 6) and subsequent application of the balance pressure an increase of the "effective" value of the MF or locomotory pressure directed from the enriched part of plasmodium is observed.

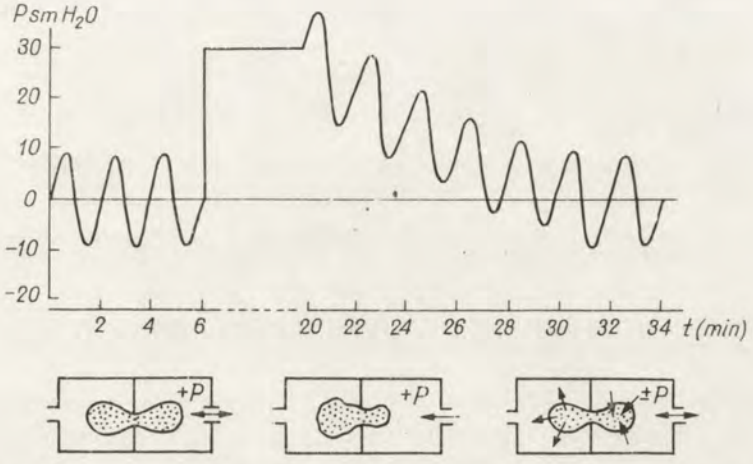

Fig. 6. The influence of the artificial polarization of the endoplasm on the motive force

The application of the balance pressure during 15 min results to a drop of the locomotory pressure up to the control level. As is evident from our model this relaxation process relates to the restoration of the membrane tension (and therefore with restoration of the volumes). Considering that protoplasmic flow is stopped by the counterpressure, it is felt that the restoration of the volumes is caused by the transport of water through the membrane (see Fig. 6). The parts of plasmodium enriched with the endoplasm and poor in it were separated, singly weighed and placed in water. The weight of the enriched part decreases after 20 min whereas that of the second part increases according to the amount of the shifted endoplasm and initial volume of plasmodium (Table 1).

A similar character of the changes in the MF of the stream on polarization has been observed by Kamiya (1968). But he has attributed it to the restoration

Table 1

The changes in relative weight of the plasmodial strands (P%) after 20 min incubation in Prescott–James solution

The endoplasm-poor strands	The endoplasm-rich strands
109	90
104	91
105	91
120	87
125	89
109	80
123	85
120	85
149	80
170	87
$P=123.4\%$	$P=84.5\%$

of the disturbed plasmagel-plasmasol equilibrium with the help of the sol-gel transition. It is possible that the sol-gel transition affects on HE – parameter. Our results indicate the important role of the osmotic (hydrostatic) water transport.

Influence of the local and general anaesthetics on the motive force

It has been shown earlier (Korohoda 1969) that treatment of plasmodium by the local anaesthetic benzamide (10–50 mM) results in an intensive influx and accumulation of great masses of protoplasm in the treated region. We found the same effect using also general anaesthetics urethan (2.5 per cent) and hexenalum (40 mM).

The influence of urethan at a concentration of 2.5 per cent on the change of the value and polarity of the locomotory pressure is shown in Fig. 7. This pressure at first acts towards the compartment 1 (control). The local treatment of the plasmodium in compartment 2 caused a sharp change in polarity and increase of locomotory pressure which should result from decrease of the tension in the treated part of plasmodium.

Figure 8 shows a similar change in a locomotory pressure under the action of benzamide at a concentration of 25 mM (only the "polar line" is presented in this figure). The addition of $CaCl_2$ (0.5–1 mM) diminishes or neutralizes completely the effect of the anaesthetics (Layrand and Matveeva 1971).

The mechanism of relaxation of the cortical layer of plasmodium under the action

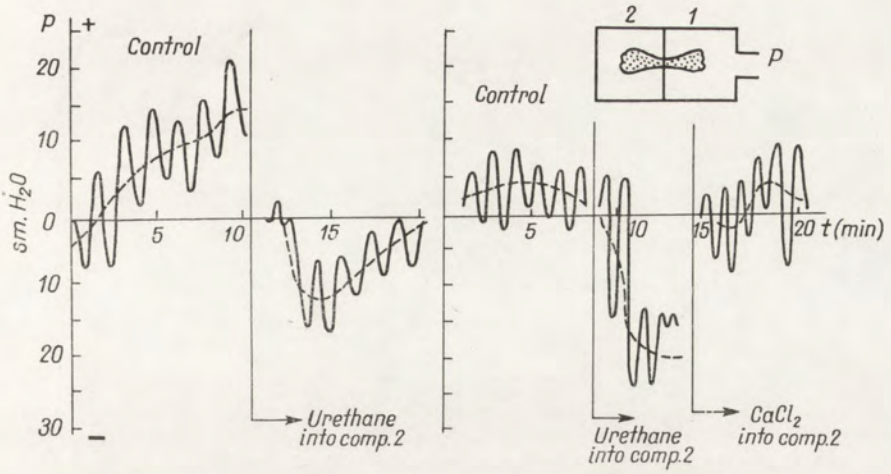

Fig. 7. The change of the motive force and the locomotory pressure under urethane influence (2.5%). The dotted line shows polarity line of the motive force

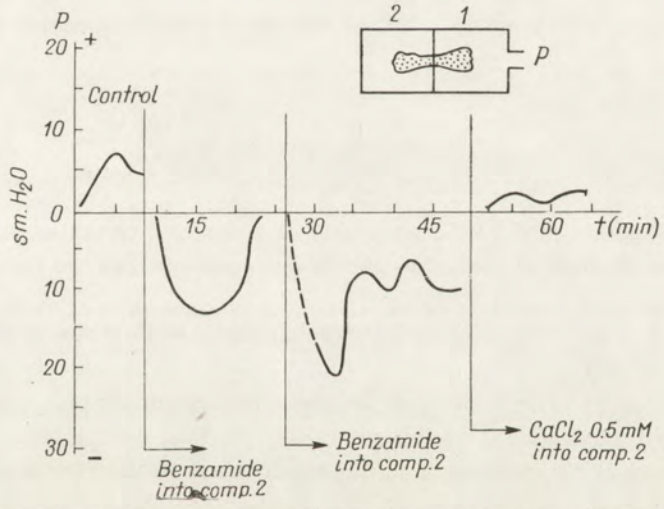

Fig. 8. The influence of benzamide (25 mM) on the locomotory pressure

of anaesthetics is not clear. Their action seems to be based on the competition with calcium and on the removal of calcium from the membrane and or peripheral gel network. The relaxation effect, though not so well pronounced, can be observed by treating the plasmodium with EDTA or sodium citrate (Layrand 1968). The role of the relaxation in the cell locomotion at the normal conditions is demonstrated

Fig. 9. The changes of the microplasmodia contours (1–10) during his locomotion registrated at 30 sec intervals

on Fig. 9. There are slow severe periodical relaxations and contractions of the plasmodium envelope along with minor changes caused by the shuttle-streaming of the endoplasm. This type of locomotion resembles the locomotion of the rain-worm.

Concluding remarks

The data obtained confirm the possibility of generation of the movement in the cell by periodic changes of the value *HE* as was considered in the theoretical part of the paper.

It should be particularly emphasized that changes in the thickness of the envelope, for example, gel-sol transitions observed in the cell during the amoeboid movement which are considered to be responsible for the movement of the protoplasm (Lewis 1942) also reflect the change of this parameter. In this system great importance in the regulation of the movement must be attributed to the electric fields in the membrane and to the permeability of the membrane to water and ions since they must influence the state of the polyelectrolytic gel adjacent to the membrane. It is necessary to note induction of pseudopodia in amoeba by the local action of different polycations neutralizing negative superficial charges of the cell membrane (Brewer and Bell 1969, Bingley et al. 1962). The existence of the positive intracellular pressure suggests that the membrane should be "closely connected with the structurized gel layer". The available data enable one to consider the membrane with the adjacent gel layer, when the intracellular pressure exists in the complex as a unified receptor-effector system.

Summary

Theoretical analysis of the elastic spherical osmotic cell has been made and the dependence of spherical osmotic cell volume on the molal concentration of the medium is given. The dependence received in experiments with plasmodium myxomycete *Physarum polycephalum* coincides well in range of the methods with the theoretical one for certain parameters. The coincidence allowed to obtain characteristic parameters of plasmodium: (1) internal osmomolality C_i(70 mosM/kG H_2O); (2) intracellular hydrostatic pressure P (1.7 atm.); (3) "osmotic dead space" V_d (17.4%); (4) the value HE (5×10^6 din/cm^2), where E is the modulus of elasticity of superficial layer and H—its relative thickness. Experiments with endoplasm rich and endoplasm-poor plasmodia showed that the cell maintains the optimal internal pressure by osmotic forces. The hypothesis is given on the role of the intracellular pressure in cell movement. The tension gradient of superficial layers which arises under local changes of HE values is believed to be the motive force of protoplasmic flow and cell locomotion. The assumption is confirmed by the observations that local relaxations of superficial layers of plasmodium induced by anaesthetics cause corsponding changes of direction of locomotory pressure.

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Local contraction-relaxation processes in Amphibian eggs induced by AC-electric fields

It is a well known phenomenon that certain electric stimuli applied to living cells induce contraction of the cytoplasm, followed in turn by spontaneous relaxation. Amoebae subjected to a direct current electric field after a few minutes usually round up and stop locomotion (Daniel and May 1950). Similarly, amoeba exposed to alternating current electric fields contract at the regions directed towards the electrodes and produce only pseudopodes oriented perpendicularly to the lines of the electric field (Mast 1931). However, if the current is switched off, new great pseudopods are formed exactly at the regions that were formerly induced to contract (Korohoda, unpublished).

Processes of contraction and relaxation of the cellular cytoplasm are supposed to play a basic role not only in protoplasmic streamings, and cellular locomotion, but – as it has been repeatedly stressed – also in cell division (cf. Marsland 1956, 1970, Wolpert 1960, 1963). Since it was possible to stimulate, at least in some degree, contraction and relaxation processes in amoebas, an attempt was made to induce similar responses in amphibian eggs.

Since the processes occurring during normal cell division are supposed to have a bipolar character, it was decided to subject the eggs to the action of an alternating current (AC) – electric field of a frequency of 50 Hz.

All experiments were performed in an experimental chamber made of plexiglass (Fig. 1). The chamber was composed of the main chamber (MC) containing two silver/ /silver chloride reversible electrodes (E), and of a small compartment (SC) lined with a mixture of paraffin and beewax (PW). In this lining one or two cavities were made in which the eggs were positioned. The latter chamber (SC) was exchangeable, which enabled faster manipulation of the eggs during successive observations.

As material for the experiments eggs of the South-African clawed toad *Xenopus laevis* were chosen. In order to enable exact timing of the material the eggs were inseminated in vitro (for methods see Rzehak 1972), and allowed to develop at 17–18°C. At this temperature the first cleavage furrow appears about 90 to 100 min

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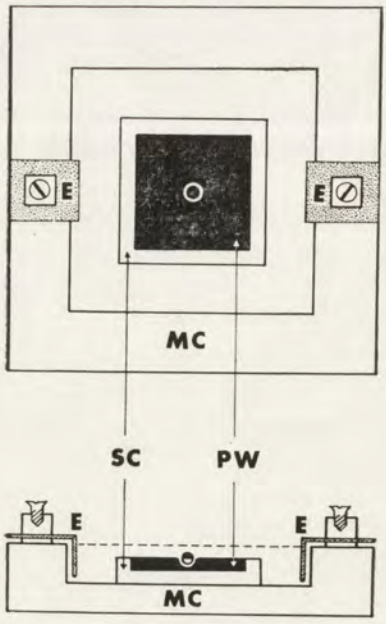

Fig. 1. Experimental chamber, vertical view, and below—horizontal cross-section. MC—main chamber; SC—small chamber; E—silver/silver chloride reversible electrodes; PW—paraffin-bee wax lining

after insemination. All observations were performed on eggs from which most of the jelly was removed mechanically. As the medium for the experiments a full strength or 1/10 Holtfreter solution was used.

On the basis of first introductory observations two conclusions could already be drawn:

(1) The responses of the eggs, like in amoebae (Korohoda and Kurowska 1970), depended upon the voltage gradient in the medium and not upon the density of the current flowing through the medium. Therefore, if two solutions were used, one differing in concentration, and thus in electric conductivity, from the other, two different densities of the current had to be found in order to evoke the same kind of stimulation.

For example, the current density which stimulated the egg to react had to be 10 times higher in a full strength Holtfreter solution than in the 1/10 Holtfreter medium. The same voltage gradient, however, caused always the same particular cell responses, independently of the concentration of the medium.

Cell reactions could be evoked only at gradients varying from 3–5 V/cm. Gradients below this value, even if applied for 10 min or more, did not induce any responses. On the other hand gradients higher than 5 V/cm caused as a rule the break of the egg membrane followed by the formation of extensive extraovates, and — as a consequence — by cell death. Since gradients of 4–5 V/cm, if applied for more than 2–3 min, could also cause injuries, the observations were finally restricted to eggs subjected to AC fields of 3.0–3.5 V/cm, for no more than 1 min. Most of the observations were carried out on eggs placed in a full strength Holtfreter solution.

(2) The second conclusion which could be drown from the introductory experiments was based on the observation that the responses of the eggs to a given stimulus changed with the time which passed after their fertilization. Therefore it was very important to determine in the successive experiments the age of the eggs used.

In the responses of the eggs to electric stimuli three main reaction patterns could be distinguished:

The eggs examined 15 to 50 min after fertilization (times from 0 to 15 min were not examined because of fertilization procedure), when subjected to the AC-field, (Pl. I 1–4), react by a slight flattening of the regions pointed towards the electrodes. At these regions, too, a part of the pigment accumulates, which is visible up to 2 min after the current has been switched off.

Thereafter the shape of the egg becomes spherical again, and the pigment distribution returns to its original pattern. Occasionally the pigment accumulation and the flattening of the regions pointed towards the electrodes are accompanied by the appearance of wrinkles which radiate from the most heavily pigmented area (Pl. I 5).

The reaction of the eggs stimulated 50–85 min after fertilization (Pl. I 6–10) is similar, but much stronger. The pigment accumulation which persists for 1–2 min after switching off the current, is particularly evident. However, soon the strongly pigmented areas start to swell, forming clearly visible bulges. After subsequent 10–15 min the eggs resume their original spherical shape, though the pigment pattern may still show certain abnormalities. Eggs stimulated in this way cleft normally later, at the same time as control eggs.

The most spectacular and characteristic responses are observed in eggs stimulated during the last 10–15 min before the onset of their normal cleavage, i.e., 85–100 min after fertilization (Pl. II 1–12). Although pigment accumulation and sometimes also flattening of the induced poles are visible, they are usually less distinguishable than in eggs induced earlier. About 2 min after the current has been switched off the bulging out of the pigmented areas takes place again. Shortly later, however, a ring-shaped constriction appears, at the base of the forming bulges, giving rise to the formation of clearly visible furrows. During this process the pigment shifts partially towards the bottom of the furrows. While deepening the furrows usually travel in the direction of the middle of the egg, and sometimes join.

The induced furrows (or the "joint" furrows) seem to be morphologically indistinguishable from normal cleavage furrows. However, after they have existed for 2 or 3 min they disappear, never leading to a permanent separation of the "pseudoblastomers". Instead, during the next couple of minutes normal cleavage begins, whereby the plane of the natural furrow shows no relation to the position of the induced furrows. The natural furrow can go practically in any direction, being apparently determined only by the position of the mitotic apparatus.

The observations described above show that in the amphibian egg, similarly as in amoebae, the cell membrane may be stimulated electrically. The direct result

of this stimulation is local pigment concentration, flattening of the stimulated regions, and appearance of wrinkles running radially from the flattened areas. This suggests that electric stimulation brings about local contraction of the regions directed towards the electrodes. After the current is switched off, the formerly contracted areas bulge out, which may be considered as a result of their simultaneous relaxation. It should be emphasized, however, that electric stimulation results primarily in local contraction, which only later is followed by a chain of events occurring long after the current has stopped to flow. Electric stimulation seems therefore to set off only certain intrinsic cell reactions and not to act as a factor providing the forces for the described changes in the egg.

In order to obtain reproducible reactions of the egg it was necessary to control the electric field, i.e., the voltage gradient in the medium, and not the density of the current flowing through the medium.

The egg was able to react only if a certain threshold value of the voltage gradient was overstepped, and it is worth mentioning that this threshold was of the same order of magnitude as in amoebae (Korohoda and Kurowska 1970).

Not much can be said at the moment about the mechanism of the described processes. One can expect, however, that the stimulation of the egg by an electric field should increase locally the permeability of the cell membrane. This in turn could result in the ensuing of ionic fluxes evoking the observed local contraction of the stimulated regions. It may be mentioned at this place that similar contractions accompanied by pigment concentration and appearance of wrinkles can be produced if highly charged polycations are applied locally to the egg surface (Gingell 1970).

Although the bearings of the described observations upon contemporary theories of cytokinesis will be described elsewhere, one point should be stressed already now. In eggs which are close in time to normal cell division relaxation of formerly contracted regions is followed by a contraction in the area which was not stimulated before. This second contraction leading to the formation of artificial furrows takes place only during the last 10–15 min before the onset of normal cytokinesis, which suggests that the superficial layers of the cell are able to react with furrow formation exclusively during a certain time interval. Cell division is always preceded by a gradual increase in the rigidity of the cell ectoplasm (cf. Mitchison and Swann 1955, Selman and Waddington 1955, Marsland 1956, Wolpert 1960, 1963) which probably results in a general contraction of the superficial layers of the cytoplasm. The results presented above confirm the idea that contraction preceding cell division may be a prerequisite of furrow formation. As it has been stressed, induced furrows appear only after relaxation of formerly stimulated areas. One can expect therefore that the contraction in the division plane leading to the formation of cleavage furrows is the final result of an interplay of the general contraction of the cell and of local relaxation.

Although further experimental proof is needed, the observations described

in this paper seem to provide strong support for the cortical gel contraction theory of Marsland (1956, 1970), and the astral relaxation theory of Wolpert (1960, 1963).

Summary

Fertilized *Xenopus* eggs were subjected for 1 min to AC-electric fields of a frequency of 50 Hz, and a gradient of 3.0–3.5 V/cm. The responses of the eggs depended upon their age, that is the time which lapsed from fertilization until the action of the electric current. Most spectacular changes were observed in eggs stimulated during the last 50 min before the onset of their first normal cleavage. At the poles of the egg directed towards the electrodes, clearly visible contraction takes place, which still continues after the current is switched off. This contraction, accompanied by pigment concentration, is followed by bulging out—interpreted as relaxation of the formerly contracted areas. In eggs stimulated 10–15 min before normal cytokinesis, at the base of the described bulges these arise furrows which are morphologically indistinguishable from normal cleavage furrows. These induced furrows disappear after 2 or 3 min and normal cell division begins. The plane of the natural cleavage furrow shows thereby no relation to the position of the induced furrows.

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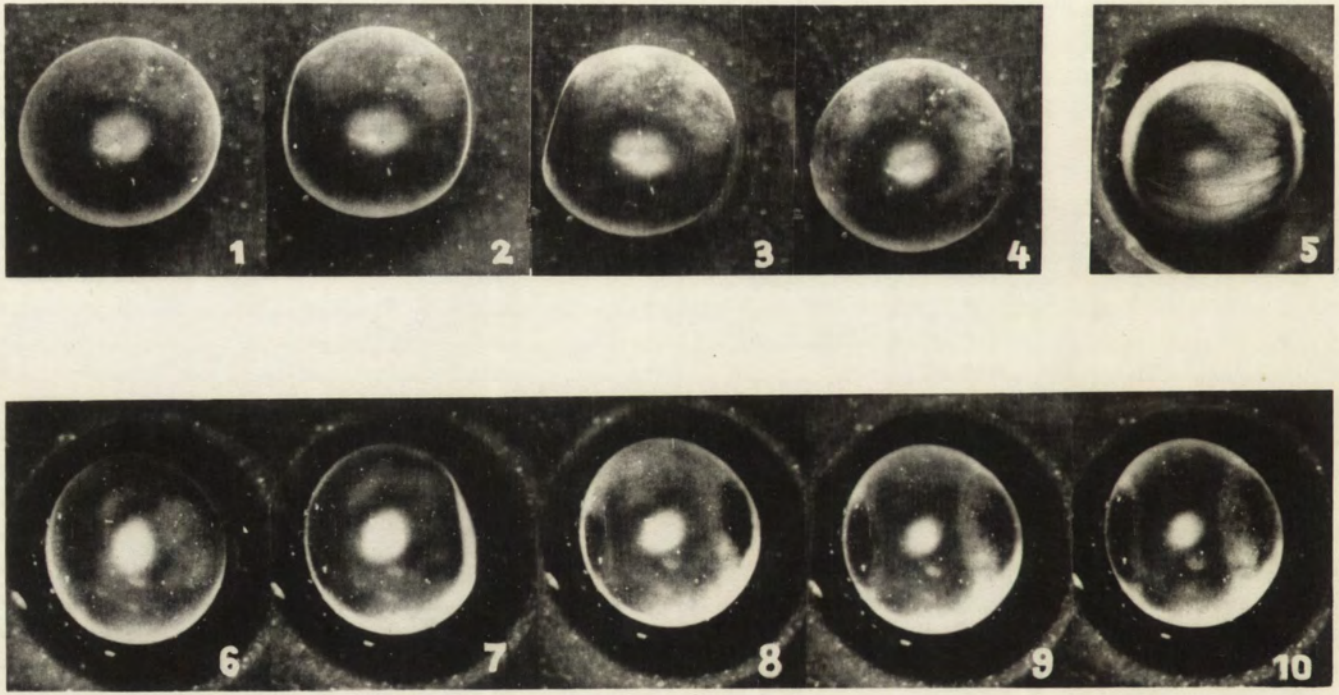
EXPLANATIONS OF PLATES I–II

Plate I

1-4: Successive stages of the responses of a *Xenopus* egg subjected for 1 min to the action of an AC-electric field 20 min after fertilization. 1—egg before stimulation; 2—egg during stimulation; a slight flattening of the poles directed towards the electrodes in seen; 3—egg 1 min after stimulation; further flattening of the poles directed towards the electrodes; at these regions also a part of the pigment accumulates; 4—egg 4 min after stimulation; the shape of the egg becomes spherical again and the pigment distribution returns to its original pattern
5: Another egg subjected to the action of an AC-electric field 25 min after ferilization; flattening of the poles and local pigment concentration are accompanied by the appearance of wrinkles
6–10: Successive stages of the responses of a *Xenopus* egg subjected to the action of an AC-electric field 80 min after fertilization; 6—egg before stimulation; 7—egg during stimulation; flattening of the poles and pigment accumulation at the regions directed towards the electrodes are visible; 8–10—photographs taken 3, 6, and 9 min after stimulation; gradual extension of the pigment formerly concentrated, suggesting that the pigment areas swell and form bulges

Plate II

1–12: Successive stages of the responses of a *Xenopus* egg subjected for 1 min to the action of an AC-electric field 90 min after fertilization. 1—egg before stimulation; 2—egg during stimulation; pigment accumulation and flattening of the poles directed towards the electrodes are visible; 3–4—egg 2 and 4 min after stimulation; pigment extension and bulging out of the regions formerly contracted; 5–6—egg 5 and 6 min after stimulation; at the base of the forming bulges a constriction appears giving rise to the formation of induced furrows; 7–8—egg 7 and 8 min after stimulation while deepening induced furrows gradually join; 9–10—egg 9 and 10 after stimulation; gradual disappearance of the "joint" furrow; 11–12—egg 101 and 104 min after fertilization (11 and 14 min after stimulation); formation of a normal cleavage furrow; the pigment distribution altered during the formation of induced furrows is still visible



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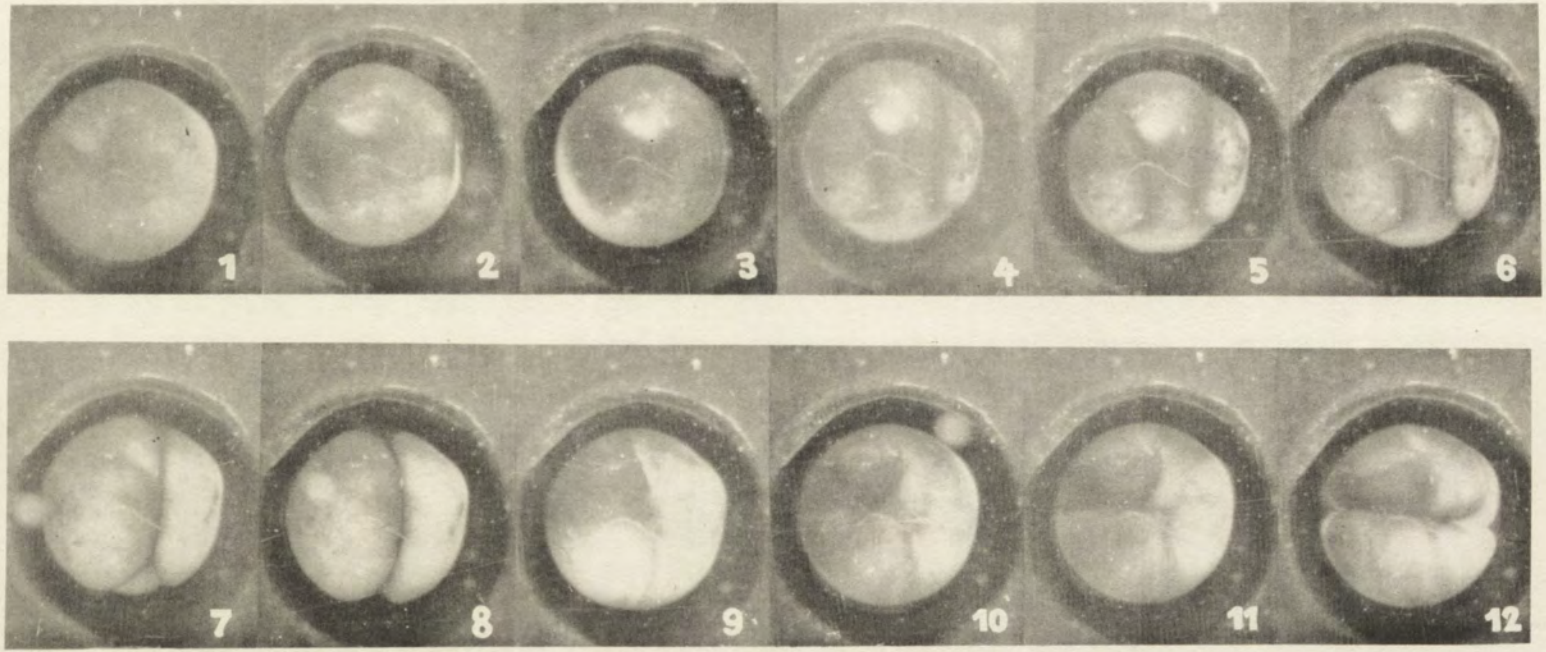

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Avoiding reaction and receptor mechanism in *Protozoa*

The so-called avoiding reaction commonly known in *Paramecium* can be elicited by various kinds of stimuli. Heat, chemical, mechanical, and ultraviolet light stimulation applied to the anterior end of *Paramecium* leads to a similar behavioral response: backward swimming caused by a transient ciliary reversal and restored forward locomotion in a deviated direction. Other protozoa exhibit corresponding responses when stimulated locally or over the entire surface. *Opalina* shows a transient ciliary reversal, which changes the direction of the rotating movement, and *Euplotes* shows a fast alternation between characteristic reversal responses, which are similar in *Stylonychia*. Machemer 1965 found that these "reversal movements" are caused by synchronous beats of all cirri.

Reversal responses are closely correlated with transient depolarizations of the cell membrane. This seems to be valid in all species investigated in this respect. There is some evidence that depolarization occurs first and initiates ciliary reversal (Kinosita, Murakami and Yasuda 1965). Naitoh and Eckert 1969 a investigated the ionic mechanism which controls the avoiding reaction in *Paramecium* and demonstrated a Ca^{++}-dependence of the receptor potential. Their most probable assumption is that the membrane conductivity to Ca^{++} transiently increases and permits a calcium influx. The depolarization caused spreads electronically over the whole cell membrane. These findings are an important step in exploring the receptor mechanism in protozoa. The molecular mechanism of stimulus reception, however, remains unsolved. One of the most important questions is: What are the mechanisms which lead from the reception of stimulus energy to the change of cation-specific membrane permeability? The fact that protozoa exhibit their characteristic avoiding reactions due to ultraviolet light stimulation enables us to get some information about cellular substances, which are involved in the transducer process.

In this respect some experiments were made with *Opalina ranarum* and *Euplotes vannus*. Intracellular recordings were obtained from *Opalina* and *Euplotes* in standard solution (Hildebrand 1969, see also for immobilization technique) by means of 0.5 M KCl-filled capillary electrodes of about 10 megohm resistance. Membrane resistance was measured in *Opalina* by two inserted microelectrodes using the square

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pulse technique. Ultraviolet light of different wavelengths was generated by a 200 W super pressure mercury source in connection with a grating monochromator (Bausch and Lomb). The incident intensity was in the order of 100 erg $\times$ mm^{-2} $\times$ s^{-1}. Another series of experiments concerning the spectral sensitivity of *Euplotes* was made by measuring the latency of the avoiding reaction using a stop watch and microscopical observation. In all experiments the whole cell was irradiated.

As responses to ultraviolet light stimulation transient membrane depolarizations in the range of normal spontaneous potential changes were measured both in *Opalina* and *Euplotes*. It is known from the investigations of Naitoh and Eckert 1969 a, b that membrane depolarization is elicited by stimulation of the anterior end of the specimen, whereas stimulation of the posterior end causes hyperpolarization. Their findings agree with behavioral studies made by Jensen 1959, who used UV-microbeam stimulation. Thus irradiation of the whole cell will produce a resultant response of two opposite effects.

The maximal amplitude of elicited depolarizations in *Opalina* was about 6 mV (Fig. 1). The steepness of potential change is dependent on stimulus intensity and

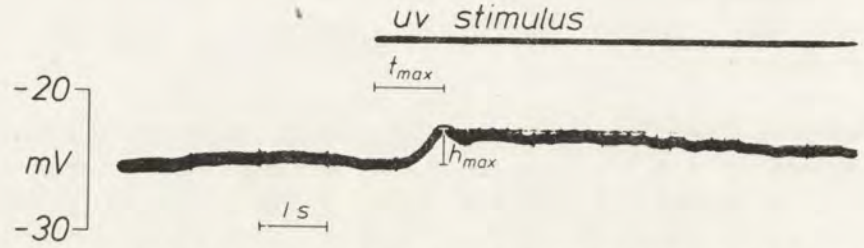

Fig. 1. Membrane depolarization in *Opalina* due to ultraviolet light stimulation (wavelength 249 nm, intensity approx. 100 erg $\times$ mm^{-2} $\times$ s^{-1}), h_{max} = maximal amplitude of depolarization, t_{max} = time from the beginning of the stimulus to the peak of the response

wavelength. $\frac{h_{max}}{t_{max} \times I_{rel}}$ (I_{rel} = relative intensity, for h_{max} and t_{max} see Fig. 1) therefore gives a relative measure of stimulus efficiency. The latency was very difficult to measure exactly because of spontaneous fluctuations of the membrane potential. It was in the order of 0.3 sec and seems to be rather independent of intensity. Maximal depolarization was always correlated with ciliary reversal.

Euplotes exhibits all-or-none responses, which are phases of strong depolarization to about 10 mV lasting several seconds (Fig. 2, upper graph). They are similar to spontaneously occurring depolarizations and it has been shown that they are most probably closely correlated with the avoiding reaction (Hildebrand 1970, see also Machemer 1970 for *Stylonychia*). The latency of both the electrical and behavioral response is dependent on stimulus intensity and wavelength. Similar depolarizations can be elicited by intracellularly applied depolarizing currents of several nanoamperes

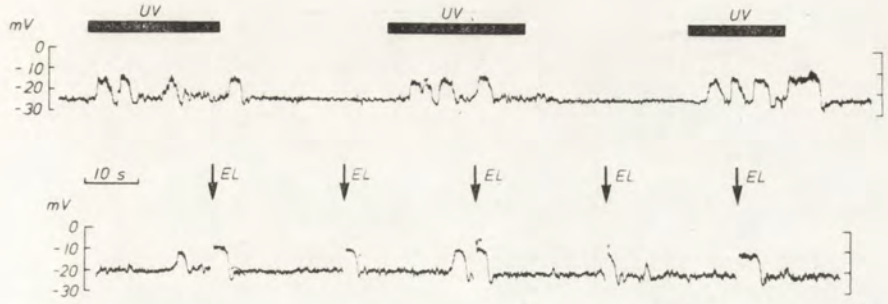

Fig. 2. Membrane depolarizations in *Euplotes*. Upper graph: Elicited by ultraviolet light (UV) stimuli (wavelength 249 nm, different intensities). Lower graph: Elicited by intracellularly applied depolarizing currents (EL) of several nanoamperes

(Fig. 2, lower graph). This fits well to the observation that *Euplotes* shows a fast series of reversal responses in the electric field.

Decrease of membrane potential is accompanied by a decrease of membrane resistance as well in spontaneous as in stimulus-induced excitation. This could be demonstrated with *Opalina* (Fig. 3). Following the conclusions of Naitoh and Eckert

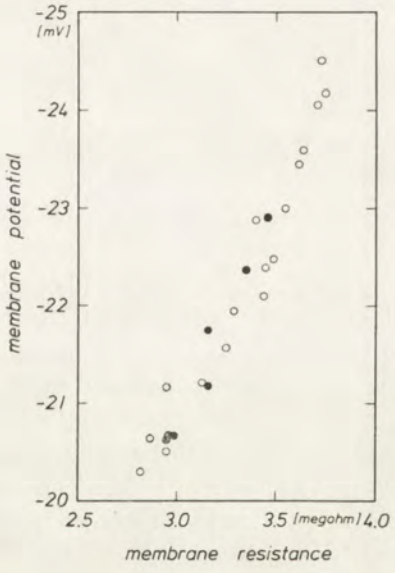

Fig. 3. Dependence of the membrane potential on the membrane resistance in *Opalina* during spontaneous excitation (open circles) and during UV stimulation (closed circles). Temp. 16–18°C

1969 a one can assume an increased membrane conduction to Ca^{++}. The action spectrum obtained from *Opalina* (Fig. 4) shows some similarities with absorption curves of common proteins (for instance egg albumin) with respect to maxima and minima. The curve of spectral sensitivity obtained from *Euplotes* (Fig. 5) permits a more exact comparison with the absorbancy of cellular constituents. The curve

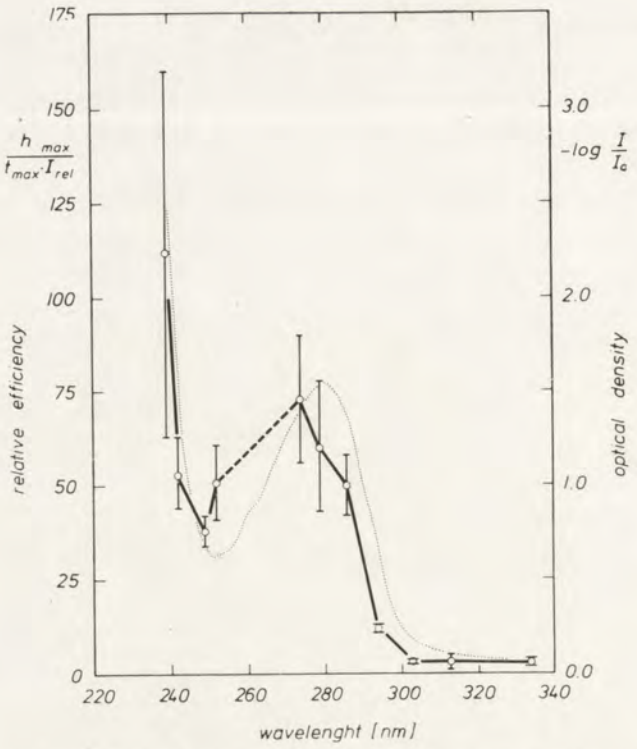

Fig. 4. Action spectrum for the transient membrane depolarization in *Opalina* (left ordinate) Each point represents the mean of at least 3 measurements. Vertical lines give the standard error of the mean. Temp. 17–20°C. The dotted line represents the absorbancy of 1 cm layer of an aqueous solution of 0.2 per cent egg albumin (right ordinate)

shows a second maximum about 260 or 265 nm and a relatively strong sensitivity about 250 nm with respect to the height of the 280 maximum. At a glance it seems to be obvious to assume a nucleotide taking part in the process of excitation; the role of nucleotides, however, must be negligable because of their strong absorbancy (see McLaren and Shugar 1964, p. 79).

The suspicion that the membrane is affected by heat due to the UV-absorption by the cytoplasm or by the whole cell can be refuted by a simple calculation. Local heat effects due to UV-absorption by the membrane itself, however, cannot be completely excluded.

If we assume a protein acting as the primary acceptor we have to consider the possibility that certain constituents of the protein contribute to the effect in a particular way. It was found by Dose and Rajewsky 1961 that particularly disulfide-bonds are strongly sensitive to ultraviolet radiation and it is cystine which shows a relative strong absorbancy in the range of 250 to 260 nm (Setlow and Doyle 1957). This effect could possibly explain the spectral sensitivity curve. But if this

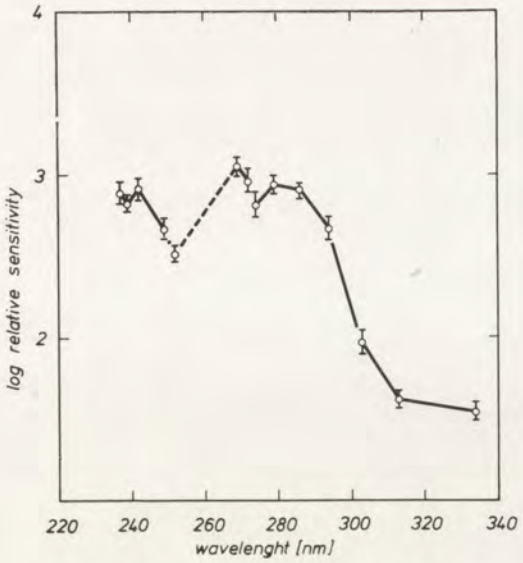

Fig. 5. Spectral sensitivity of *Euplotes* with regard to the avoiding reaction. Ordinate: Logarithm of the reciprocal of relative numbers of quanta needed to elicit the response 7 sec after the beginning of irradiation. Each point represents the mean of 15 measurements. Vertical lines give the standard error of the mean. Temp. 20–24°C

hypothesis holds true, what is the mechanism by which the Ca^{++} conductivity is increased? The acceptor protein can be assumed to be either part of the cell membrane itself or to be closely related to the regulation of the membrane permeability. There are at least three possible explanations:

(1) The protein component of the cell membrane undergoes a conformational change possibly due to rupture of disulfide linkages, which directly leads to the increased conductivity.

(2) It seems possible that Ca^{++} bound to anionic sites, which makes the membrane tight, is liberated. This might perhaps happen in connection with conformational changes and would agree with the ion exchange properties of the protozoan membrane (Naitoh and Yasumasu 1967, Naitoh and Eckert 1968).

(3) The activation of an ATPase-like enzyme, which controls the passive transport of Ca^{++}, cannot be excluded. This idea would agree with the hypothesis of Bowler and Duncan 1967. Membrane ATPases are known to be activated by SH-groups (for review see Duncan 1967). Thus activation might take place by SH-groups appearing as a result of photolysis of disulfide linkages.

At present we cannot favour one of these speculations. But further experiments testing the different hypotheses may help to understand the receptor mechanism on the molecular level. Protozoa seem to be suitable objects for such investigations because of their rather unspecific and presumable basal receptor properties.

Summary

Transient membrane depolarizations resp. reversal responses can be elicited by ultraviolet light stimulation applied to the whole cell of *Opalina ranarum* and *Euplotes vannus*. Spontaneous and UV-induced depolarizations are correlated in the same manner with an increase of permeability. The spectral efficiency of the electrical response in *Opalina* as well as the spectral sensitivity of the avoiding reaction in *Euplotes* corresponds quite well with absorption curves of proteins. The action spectra are discussed with respect to various possibilities of the molecular receptor mechanism. A protein is assumed to be responsible for the reversible change of membrane conductivity to Ca^{++}.

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Effects of detergents on excitability and motor response in protozoa

It is well known from studies on microorganisms that toxic effects of detergents depend mainly on the deterioration of cell wall or plasma membrane due to their affinity for lipid and lipoproteid components of membranes. So far scarce information is available about action of the surface active substances on the cell excitability and motility. Butzel et al. 1960 noticed the inhibiting effects of cationic detergent cetyl trimethyl ammonium bromide (CTAB) on galvanotactic response in *Paramecium* whereas Webb 1964 reported that CTAB causes decrease in twich tension of isolated frog sartorius muscle. Cationic detergents are known also to decrease the demarcation potential of sartorius muscle in frog (Wassano et al. 1956) and resting potential in the squid axon (Kishimoto and Adelman 1964).

It was found recently by the authors (Bujwid-Ćwik and Dryl 1971) that in the ciliate protozoa detergents may evoke a number of motor response such as continuous ciliary reversal (CCR), periodic ciliary reversal (PCR), or single short-lasting reversals of ciliary beat. The rate of forward movement is usually decreased, although in some cases (e.g. in *Spirostomum ambiguum* exposed to Tween 80) increase of swimming rate was noticed during first minutes of exposure.

The intensity of motor reactions induced by detergents depends on their concentrations. Long lasting CCR response followed by slackening of forward movement appears usually in toxic, sublethal doses of detergents while PCR response or single sporadic reversal responses were more or less typical reactions in lower concentrations.

So far no relation could be found between the electric charge of detergent and the kind of induced motor reaction; the slackening of forward movement, CCR and PCR appeared in anionic, cationic and non-ionic detergents as well. However, it should be added that the same detergent may evoke quite different motor reactions and there are also significant differences in toxic effects and deterioration of pellicle and cell membrane in various detergents.

Almost all detergents when applied in toxic, sublethal doses cause changes in the

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shape of the animal and in contraction phenomena within ectoplasm and myonemes. Typical contractions were observed in *Spirostomum* and *Stentor* after exposure to sodium dodecyl sulfate and CTAB. In *Euglena gracilis*, sodium dodecyl sulfate and CTAB caused metabolic movement, whereas retraction of pseudopods was observed in *Amoeba proteus*.

Another important question is whether surface active substances can influence the level of cell excitability expressed in changes of threshold level intensity of response to external stimuli. The preliminary observations of the authors revealed that some cationic detergents (CTAB, Sterinol, Laurosept) inhibit K^+/Ca^{++} – induced CCR response in *Paramecium caudatum*, while anionic ones (sodium dodecyl sulfate) strengthen this response as could be concluded from its longer duration.

The authors decided to carry out more extensive studies in this respect on *P. caudatum* since this species proved to be very sensitive to action of detergents and because a large body of information was already available on response of this ciliate to various external stimuli.

Material and methods

The experiments were carried out on *Paramecium caudatum* which was cultivated in lettuce medium inoculated with *Aerobacter aerogenes* according to method of Sonneborn 1950. Before starting the experiments paramecia were washed with 1 mM $CaCl_2$+1 mM Tris-HCl buffer solution of pH 7.4 in which they were kept for 12–18 h. All tested chemical agents (CTAB, KCl, $BaCl_2$, quinine etc.) were diluted with the same $CaCl_2$-Tris-HCl buffer solution.

The behaviour of paramecium was observed directly in deppresion slides with 0.4 ml of medium by means of low power binocular microscope. The duration of ciliary reversal response in *Paramecium* was calculated on the basis of direct observations of 30–40 animals. The observed response was considered as finished when less than 50% of animals behaved in typical way.

The negative chemotactic response to various factors (40 mM NaCl, 40 mM KCl, 30 mM $MgCl_2$ and 0.1% quinine) was checked by technique described by one of the authors (Dryl 1959).

The galvanotactic response was tested in specially arranged small glass chamber, 7.5 cm long, 0.5 cm wide and 1 mm deep with two platine electrodes located at the distal ends. The response was considered as positive when more than 75% of animals were swimming towards cathode after 15 sec of continuous d.c. stimulation. The experiments were carried out at temperature 22–24°C.

Results and discussion

When paramecia are exposed to higher concentrations of CTAB in Ca-Tris-HCl medium they swim slowly forwards along large amplitude spirals or arcs and during the first seconds of exposure the shape of body is changed into a characteristic pear-like form with the thickening of the anterior and contraction of the posterior part of the body. The longitudinal axis of the animal is shortened while the transverse

one is elongated when compared to the normal, unexposed animal. After 20–30 sec of exposure the rate of forward movement is further slowed down and almost completely stopped. At this stage the activity of contractile vacuoles and the rate of food vacuoles formation is inhibited, while the cyclosis of vacuoles and crystals in the cytoplasm is very slow, sometimes almost stopped completely in relation to the rather vivid cyclosis in thigmotactic animals.

More than 50% of animals die between 4–8 min in 1.6×10^{-5} g/ml CTAB or 15–25 min in 4×10^{-6} g ml CTAB usually with visible signs of damage to pellicle and ectoplasm, extrusion of trichocysts or vacuolisation and darkening of cytoplasm. It should be noted in this respect that paramecia can recover normal shape of body, motility and other physiological features when washed with Ca-Tris-HCl medium after first 20–30 sec of exposure even in solution with high concentration of CTAB.

In the concentration range of $1–2\times10^{-6}$ g/ml CTAB the animals show during first 30–60 sec more or less typical Periodic Ciliary Reversal (PCR) characterized by alternating short lasting periods of normal forward movement and reversed ciliary beat. When the PCR response is over, the animals swim forward again with the characteristic so-called "peripheral reaction" i.e. they swim closely to the edge of the vessel in which they were placed. At still lower concentrations of CTAB only single short-lasting ciliary reversal response appear during first 2–3 min while afterwards the animals show normal behaviour (Table 1).

Another series of experiments concerned the possible changes in the excitability level induced by detergent. Kamada and Kinosita 1940 and more recently Jahn

Table 1

Effects of various concentrations of Cetyl-trimethyl-ammonium bromide (CTAB) on *Paramecium caudatum*

Concentration CTAB	Motile behaviour of *Paramecium caudatum*		Shape of body
	during 1 min of exposure	after 1 min of exposure	
1.6×10^{-5} g/ml	slow "pivoting" movement first 10–15 sec	almost complete stopping of movement	pear-shaped deformation of body
8×10^{-6} g/ml	slow "pivoting" movement during first 20–30 sec	almost complete stopping of movement	,,
4×10^{-6} g/ml	slow "pivoting" movement during first 30–50 sec	,,	,,
2×10^{-6} g/ml	PCR during first 30–40 sec	slow movement	,,
1×10^{-6} g/ml	PCR during first 40–60 sec	normal speed of movement	,,
5×10^{-7} g/ml	single reversal responses	normal speed of movement	normal shape
2.5×10^{-7} g/ml	single reversal responses	normal speed of movement	,,
1.25×10^{-7} g/ml	single reversal responses	normal speed of movement	,,
0.62×10^{-7} g/ml	no changes in behavior	normal speed of movement	,,
Control	no changes in behavior	normal speed of movement	,,

Table 2

Response of *Paramecium caudatum* towards various external stimuli after 3 min long exposure to CTAB

Concentration of CTAB	Response to 25 mM KCl + 1 mM $CaCl_2$ (Duration of CCR + PaCR in sec)	Response to 2 mM $BaCl_2$ + 1 mM $CaCl_2$ (Duration of CCR in sec)	Response to strongly negative chemotactic agents: 0.1‰ quinine, 40 mM NaCl, KCl etc.	Threshold of galvanotactic response
1.6×10^{-5} g/ml	no response	no response	no response	
8×10^{-6} g/ml	,,	,,	,,	
4×10^{-6} g/ml	,,	,,	,,	2.0 V/cm
2×10^{-6} g/ml	16.5 ± 1.4	15″ CCR afterwards slow forward movement	,,	1.4–1.6 V/cm
1×10^{-6} g/ml	106 ± 9	20–30″ CCR afterwards weak PCR	not investigated	0.8–0.9 V/cm
5×10^{-7} g/ml	213 ± 19	15–25″ CCR afterwards PCR	,,	0.5–0.6 V/cm
2.5×10^{-7} g/ml	263 ± 19	PCR	,,	0.4–0.5 V/cm
1.25×10^{-7} g/ml	289 ± 20	PCR	,,	0.3–0.4 V/cm
0.62×10^{-7} g/ml	321 ± 28	PCR	,,	0.3–0.4 V/cm
Control	355 ± 22	PCR	100% negative response	0.3–0.4 V/cm

1962 brought evidence that duration of ciliary reversal in *Paramecium* is a good measure of excitability in this ciliate.

As indicated on the Table 2 duration of K^+/Ca^{++} – induced ciliary reversal in paramecia exposed for 3 min to various solutions of CTAB decreased in parallel to the increase of CTAB concentrations in the external medium (Dryl and Bujwid-Ćwik 1972). There was no ciliary reversal response in solutions of 4, 8 and 16×10^{-6} g/ml of CTAB, whereas in solution 2×10^{-6} CTAB there was only partial ciliary reversal (PaCR) lasting ca. 16 sec i.e., very weak reversal reaction.

Another series of experiments was carried out with Ba^{++}/Ca^{++} factor (2 mM $BaCl_2$ + 1 mM $CaCl_2$). In control animals this solution caused PCR response, known from electrophysiological studies to be accompanied by depolarizing action potential spikes (Kinosita et al. 1964). However, barium/calcium induced PCR responses appear only in paramecia exposed for 3 min to 1×10^{-6} g/ml or lower concentartions of CTAB. In the medium containing 2×10^{-7} g/ml CTAB or higher concentrations only the short lasting CCR response appears while PCR is abolished completely.

In similar way *Paramecia* transferred to higher concentrations of CTAB (2×10^{-6}

g/ml or more) showed complete inhibition of response towards strong negative chemotactic agents (40 mM NaCl, 40 mM KCl, 30 mM $MgCl_2$, 0.1% quinine).

It is interesting to note that galvanotactic response also decreases in paralel to the increase of CTAB in the medium, but it is not completely abolished even in so high concentration of CTAB as 2 or 4×10^{-6} g/ml. On general the authors were able to confirm the results achieved by Butzel et al. 1960.

According to our preliminary observations and experimental results, the characteristic for CTAB decrease of excitability to external stimuli appears also in *Paramecium aurelia*, *P. bursaria* and *Tetrahymena pyriformis*; so far the authors have not been able to demonstrate so strongly masked inhibition of response in other ciliate protozoa.

As it was reported by the authors elsewhere (Dryl and Bujwid-Ćwik 1972) the animals can recover the normal level of sensitivity to external stimuli after being washed in medium devoid of detergent. This may suggest that during the time of stay in CTAB-free medium the detergent is washed out from the cell surface to the level at which it does not affect much the state of excitability of *Paramecium*.

Additional evidence in favour of excitability changing properties of CTAB was brought by experiments in which the authors were also able to abolish within few seconds K^+/Ca^{++} induced CCR or Ba^{++}/Ca^{++} – induced PCR by exposure to $2–4 \times 10^{-6}$ g/ml concentration of CTAB. However, it should be remarked that inhibitory effects of CTAB on excitability state of *Paramecium* could be reduced or abolished by addition of Tween 80.

It is well known that detergents show high affinity to lipid and lipoprotein components of the biological membranes and at appropriate concentration they may cause their permanent demage. However, since the observed change of response to external stimuli in the present study proved to be a reversible phenomenon, the authors assume that it should be attributed to the functional state of the cell membrane and there is no reason to postulate pathological changes within those cell structures which control the motile phenomena and the level of excitability. It is suggested that the cationic detergent CTAB may block the cell membrane chemoreceptors which in accordance with the generally accepted view can be identified with the postulated calcium binding sites (Jahn 1962, Grębecki 1964, 1965, Dryl and Grębecki 1966, Kuźnicki 1966, Naitoh and Yasumasu 1967, Naitoh 1968) and in this way the cations and some other external agents would be prevented to induce the expected ciliary reversal response in CTAB – exposed animals. It is interesting to point out in this connection that Walsh and Lee 1962 postulated blocking effects of CTAB on the conduction of impulse along the giant fibres of squid axon.

The present study is only the first step in our attempt to elucidate the mechanism of detergent action on excitability and function of locomotor organelles of protozoa. It is believed, however, that extensive studies on electrophysiology, permeability and electronemicroscopy of cell membrane and ciliary apparatus of protozoa will supply new interesting data in this field.

Summary

Cationic detergent cetyl trimethyl ammonium bromide (CTAB) applied in sublethal concentrations evokes in *Paramecium* periodic ciliary reversal response which during first minutes of exposure is followed by the forward movement at normal or decreased rate. It was proved that depending on concentration of detergent animals exposed to CTAB may show partial or complete inhibition of ciliary reversal response towards strong chemotactic or chemokinetic agents in external medium. Blocking action of detergent on receptor sites within cell membrane is postulated.

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The stopping of ciliary movements by nickel salts in *Paramecium caudatum* : the antagonism of K^+ and Ca^{++} ions

Nickel is actively transported through the cellular membrane of *Paramecium caudatum* (Andrivon 1970). The kinetics of penetration generally obeys Michaelis law. Thus, the passage of Ni^{++} through the membrane is an enzymatic phenomenon. This basic observation will guide the studies on the antagonism of some ions towards Ni^+. So the antagonism of cobalt – which is much like nickel by its properties – is due to a competitive effect between the two ions which use the same system of penetration into the cell (Andrivon 1970). The observation of penetrating curves of Ni^{++} not hyperbolic but slightly sigmoid and of the antagonism of various ions (K^+, Na^{++}, Ca^{++}, Ba^{++}, Mg^{++}) has led us to state precisely the study of the enzymatic system responsible for the penetration of Ni^{++} and more generally of the part played by K^+ and Ca^{++} ions in the membrane of ciliates.

Material and methods

Paramecium caudatum used for the experiment belong to syngen 3 mating type 5.

Basic medium used to stop the ciliary movement is made of $2°/_{oo}$ glucose dissolved in distilled water at pH 6. Nickel, potassium or calcium chlorides are added at concentrations indicated in each table of results. They do not bring about any variation of pH or any precipitation.

The quantity of nickel necessary to stop the ciliary movement has been determined according to the method described in a previous work (Andrivon 1970).

In order to make manipulations easier by avoiding continual use of labelled Ni_{63}, the estimation of the initial speed (*Vi*) of penetration of Ni into the cell is based upon the following observations previously established (Andrivon 1970):

– At the beginning the amount of Ni penetrating into the cell per mn is constant. So the first part of the curve is a straight line.

– It is in this first part of the curve that the quantity of Ni^{++} necessary to stop ciliary movements is to be found:

$$\text{So } Vi = \frac{Q - \text{quantity of } Ni^{++} \text{ absorbed to stop ciliary movement}}{t - \text{time necessary to stop ciliary movement for a given concentration of } Ni^{++}}$$

Then we show that Q remains constant during all our experiments. We name it unit – quantity = 1, so $Vi = 1/t$.

Thus the determination of the time necessary to stop ciliary movement named resistance of *Paramecium*, is sufficient to determine *Vi*.

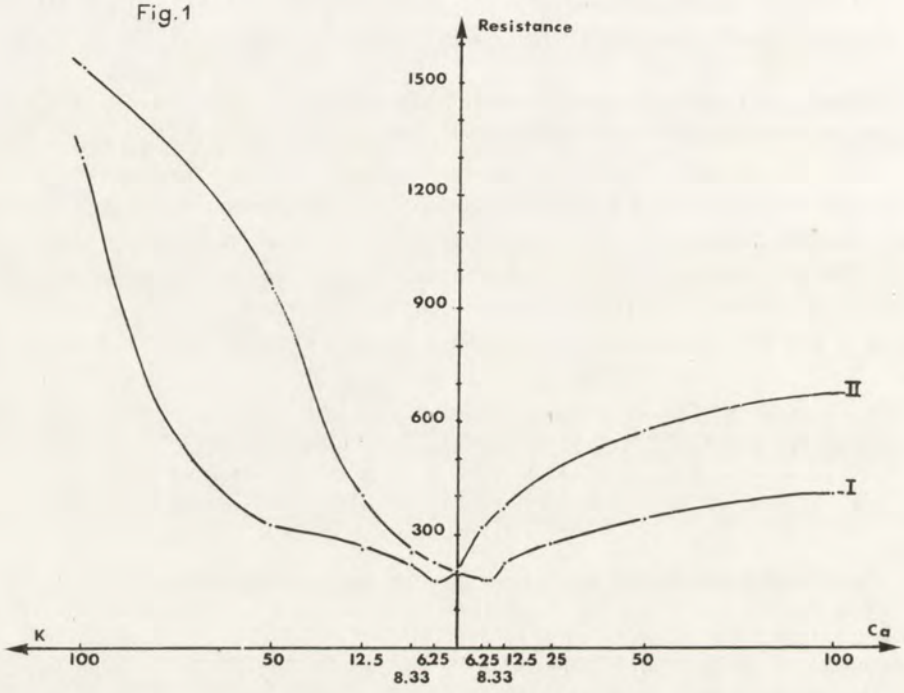
Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Experimental results

1. Influence of K^+ and Ca^{++} on the resistance to nickel salts

We have determined the resistance of *Paramecium* to a concentration of 2.5 mg/l of $NiCl_2$ $6H_2O$ in presence of increasing concentrations of $CaCl_2$ $2H_2O$ or KCl. Results are given, for two different experiments in Table 1 and Fig. 1.

Fig. 1



The results show:

(a) that calcium and potassium strongly act on the resistance of *Paramecium* to nickel salts. They either decrease it or increase it according to their concentration.

(b) that there are two kinds of *Paramecium*. We have effectively observed during our experiments, successively, and with all the possible intermediaries, populations of *Paramecium* whose resistance, under the influence of K^+ and Ca^{++}, varies as indicated by the curve I or as indicated by the curve II.

The first one named more conveniently P_{Ca} are characterised by:

— a decrease of resistance to Ni^{++} under the influence of weak concentrations of Ca^{++} followed by an increase under strong concentrations of this ion,

— a very strong increase of resistance to Ni^{++} whatever the concentration of K^+ is,

— a weak sensibility to Ca^{++}, since for equal concentration of this ion the variation

Table 1

Resistance (in seconds) of *Paramecium caudatum* treated by a concentration of 2.5 mg/l of Ni Cl_2 $6H_2O$ in presence of different concentrations of KCl or $CaCl_2$ $2H_2O$ (in mg/l)

Concentration of KCl or $CaCl_2$ $2H_2O$	Experiment 1		Experiment 2	
	resistance in presence of $CaCl_2$ $2H_2O$	resistance in presence of KCl	resistance in presence of $CaCl_2$ $2H_2O$	resistance in presence of KCl
0	203	203	201	201
6.25	177	225	314	180
8.33	177	—	—	—
12.50	224	252	366	226
25.00	272	406	—	260
50.00	344	950	575	324
100.00	405	1539	—	1299

of resistance is obviously lower than the one we have obtained with *Paramecium* defined by the curve II,

– at the same time a strong sensibility to K^+.

The later one named more conveniently P_K are characterized by:

– a decrease of resistance to Ni^{++} under the influence of weak concentrations of K^{++} followed by an increase under strong concentrations of this ion,

– a strong increase of resistance to Ni^{++} whatever the concentration of Ca^{++} is,

– a strong sensibility to Ca^{++} and at the same time a weak sensibility to K^+.

The existence of all the intermediaries between those two kinds of *Paramecium* suggests that P_K and P_{Ca} are two different states of the same system, *Paramecium* oscillating between these two states according to the amount of K^+ and Ca^{++} ions of their membrane.

We'll define this concept more precisely further on by study of variations of the initial speed of penetration of Ni^{++} under the influence of K^+ and Ca^{++} ions.

2. Studies on the quantity of nickel necessary to stop ciliary movements

In order to determine the way of action of K^+ and Ca^{++} ions upon the resistance to Ni^{++}: increase of the quantity necessary to anesthesize ciliates or decrease of the penetrating speed of ions we have measured with labelled Ni_{63} the quantity of nickel necessary to stop ciliary movements in presence of different concentrations of KCl or $CaCl_2$ $2H_2O$. Results are given in Table 2.

The quantity Q of Ni^{++} absorbed to induce the anesthesis of *Paramecium* is constant except for experimental errors. K^+ and Ca^{++} ions only act on the speed of penetration of the Ni^{++} ions. So the study of changes undergone by the enzymatic system permitting the penetration of this last one, of the competition between these three ions is essential to understand the part played by K^+ and Ca^{++} in the phenomenon of resistance to Ni^{++}.

Table 2

Quantity Q of Ni_{63} necessary to stop ciliary movement in *Paramecium caudatum* in presence of different concentration of KCl and $CaCl_2 2H_2O$

Concentrations in mg/l	Ni 2.5	Ni 2.5 + Ca 6.25	Ni 2.5 + Ca 25	Ni 2.5 + Ca 50	Ni 2.5 + K 6.25	Ni 2.5 + K 25	Ni 2.5 + K 100
Q cp/mn	178.4	171.6	187.2	169.0	174.6	180.2	167.6

3. Study of simultaneous influence of Ca^{++} and K^+ on resistance to nickel salt

Analogy between the ions (K^+, Ba^{++}, Na^+, Mg^{++}) antagonist of Ni^{++} and those bringing on ciliary reversal (Kamada and Kinosita 1940, Jahn 1962, Grębecki 1964, 1965, Kuźnicki 1965, Dryl and Grębecki 1966) has led us to examine if the two phenomenons obey the same laws in particular:

– if Ca^{++} in an antagonist of K^+,

– if K^+ acts by binding on sites usually occupied by Ca^{++} that is by decalcifying membrane. This hypothesis would permit to parallelize variations of the absorbing speed of Ni^{++} with variations of the level of calcification of the membrane, the higher permeability observed corresponding to an optimal level of Ca^{++}, and hypo- or hypercalcification leading to its decrease.

(a) simultaneous action of KCl and $CaCl_2$ $2H_2O$ on the resistance to nickel salts of *Paramecium* of P_{Ca} type.

Paramecium of P_{Ca} type have successively been anesthesized with solutions of $NiCl_2$ $6H_2O$ at 2.5 mg/l containing either:

– different concentrations of KCl or $CaCl_2$ $2H_2O$,

– a concentration of 25 mg/l of KCl and different concentrations of $CaCl_2$ $2H_2O$,

– a concentration of 50 mg/l of KCl and different concentrations of $CaCl_2$ $2H_2O$.

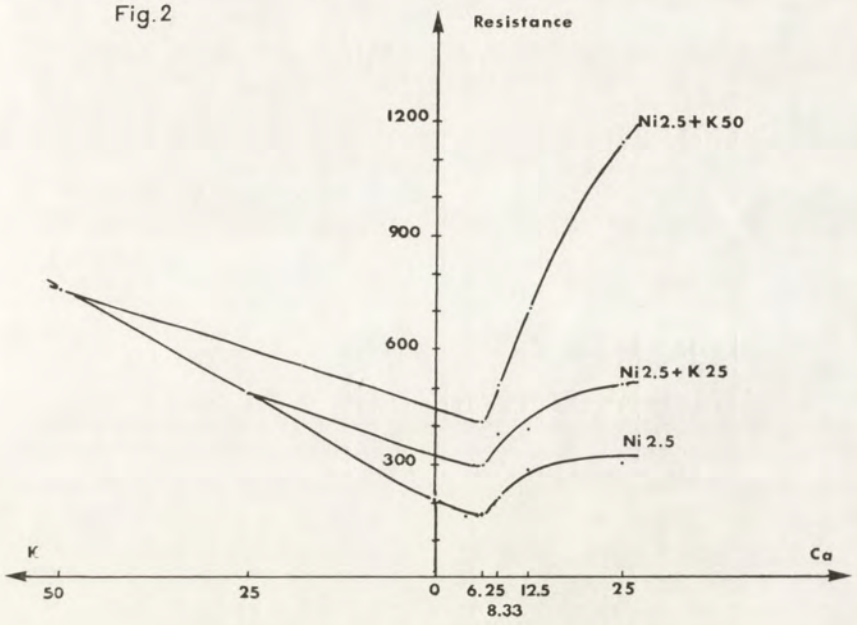
Results are given in Table 3 and Fig. 2.

The concentrations of $CaCl_2$ $2H_2O$ at 6.25 and 8.33 mg/l decrease the resistance to Ni-salts whether *Paramecium* are simultaneous treated by KCl or not. The curves showing the action of Ca^{++} are strictly parallel in the three types of experiments. In particular the greatest effect of Ca^{++} is obtained three times with the concentration of 6.25 mg/l.

When paramecium are treated by K^+ it is impossible with Ca^{++} to obtain the minimum resistance observed with *Paramecium* which have not been submitted to the influence of K^+ (168 sec). The minimum observed increases with the concentration of KCl.

With concentrations of 25 mg/l of $CaCl_2$ $2H_2O$ and 25 or 50 mg/l of KCl the effects of Ca^{++} and K^+ are added. Then the resistance is at once higher than the one observed with KCl alone or with $CaCl_2$ $2H_2O$ alone. So the antagonism of Ca^{++} towards K^+ is only to be seen in some limits of concentration.

Fig. 2



(b) simultaneous influence of KCl and $CaCl_2\ 2H_2O$ on the resistance to Ni-salts of *Paramecium* of P_K type.

The same experiments as before have been realized with *Paramecium* of P_K type. Results are given in Table 4 and Fig. 3.

The action curves of $CaCl_2\ 2H_2O$ are strictly parallel in the three types of experiments. Calcium is never an antagonist of potassium whatever the concentration

Table 3

Resistance of *Paramecium caudatum* of P_{Ca} type simultaneously treated by KCl and $CaCl_2 2H_2O$

Concentration in mg/l	Ni 2.5	Ni 2.5 + K 25	Ni 2.5 + K 50	Ni 2.5 + Ca 6.25	Ni 2.5 + Ca 8.33	Ni 2.5 + Ca 12.5	Ni 2.5 + Ca 25	
Resistance in seconds	209	484	759	168	207	282	299	
Concentrations in mg/l	Ni 2.5 + K 25 + Ca 6.25	Ni 2.5 + K 25 + Ca 8.33	Ni 2.5 + K 25 + Ca 12.5	Ni 2.5 + K 25 + Ca 25	Ni 2.5 + K 50 + Ca 6.25	Ni 2.5 + K 50 + Ca 8.33	Ni 2.5 + K 50 + Ca 12.5	Ni 2.5 + K 50 + Ca 25
Resistance in seconds	294	377	389	508	407	508	704	1149

Table 4

Resistance of *Paramecium caudatum* of P_K type simultaneously treated by KCl and $CaCl_2\,2H_2O$

Concentrations in mg/l	Ni 2.5	Ni 2.5 + K 6.25	Ni 2.5 + K 12.5	Ni 2.5 + K 25	Ni 2.5 + K 50	Ni 2.5 + Ca 6.25	Ni 2.5 + Ca 12.5	Ni 2.5 + Ca 25
Resistance in seconds	294	278	336	391	486	327	521	537
Concentrations in mg/l	Ni 2.5 + K 12.5 + Ca 6.25	Ni 2.5 + K 12.5 + Ca 12.5	Ni 2.5 + K 25 + Ca 6.25	Ni 2.5 + K 25 + Ca 12.5				
Resistance in seconds	442	605	520	652				

used to make the resistance of *Paramecium* to $NiCl_2\,6H_2O$ vary, is. The effects of Ca^{++} and K^+ are additive.

(c) Influence, on resistance to Ni^{++}, of $CaCl_2\,2H_2O$ and KCl such as

$$\frac{\text{concentration of } K^+}{\sqrt{\text{concentration of } Ca^{++}}} = \text{constant}.$$

The experiment has been performed on *Paramecium* of P_{Ca} type. We have used concentrations of $CaCl_2\,2H_2O$ belonging to the interval in which decrease of resistance to $NiCl_2\,6H_2O$ is observed that is lower than 6.25 mg/l so as to obtain the antagonist effect of Ca^{++} against K^+. Results are given in Table 5.

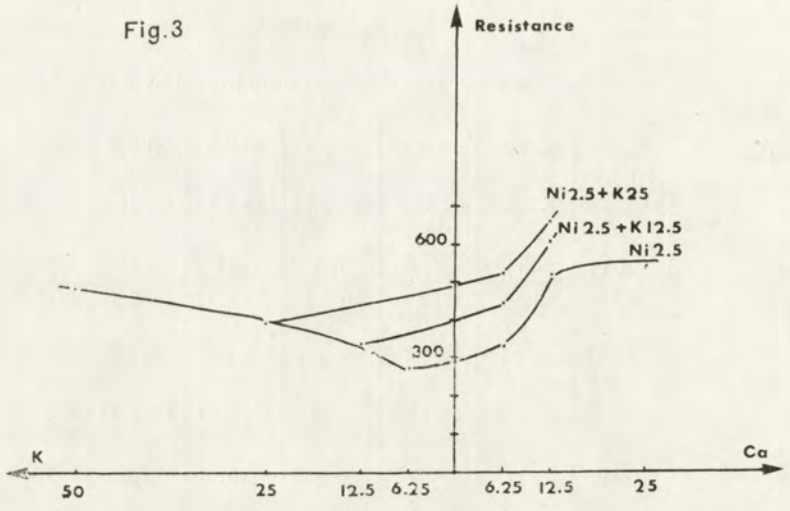


Table 5

Resistance of *Paramecium caudatum* of P_{Ca} type treated by concentrations of KCl and $CaCl_2 2H_2O$ such as $\frac{\text{concentration of } K^+}{\sqrt{\text{concentration of } Ca^{++}}} = \text{cste}$

Concentration of $NiCl_2 6H_2O$: 2.5 mg/l

Concentrations in mg/l	K 2.5 + Ca 0.312	K 50 + Ca 1.25	K 75 + Ca 2.81	K 100 + Ca 5
Resistance in seconds	322	372	726	1114

The resistance we have observed greatly varies in spite of the constancy of the ratio of concentrations of KCl and $CaCl_2$ $2H_2O$. So the effect of K^+ against Ca^{++} cannot be explained by a competition obeing a Gibbs–Donnan equilibrium.

So the three previous experiments show that K^+ does not act by decalcifying the membrane for K^+ and Ca^{++} ions are not competitive for binding at the same site and in *Paramecium* of P_K type Ca is not antagonist of K^+ does not restore the greatest permeability but in opposite it acts in synergy with it.

This observation is opposed to those made on ciliary reversal. However, the concentrations of K^+ used for our experiments are always lower than those bringing it on. So our action field is different from that of the authors previously listed who have studied the effect of ratio $\frac{\text{concentration of } K^+}{\sqrt{\text{concentration of } Ca^{++}}}$ on the duration of ciliary reversal and on the characteristics of the backward swimming of *Paramecium*: so they act after the sudden change affecting the membrane of ciliates. Does a sudden modification of the properties of sites occupied by Ca^{++} and K^+ take place then, explaining the change in the behaviour of K^+ and Ca^{++} ions towards each other?

Like other authors we have observed an antagonism of Ca^{++} against K^+ for the *Paramecium* of P_{Ca} type. But then, it seems that these two ions, though they don't enter into competition, act in opposite ways upon the penetrating system of Ni^{++}. This will be verified in the following chapters. An optimum amount of both Ca^{++} and K^+ is necessary to obtain the lower resistance. *Paramecium* of P_{Ca} type have an optimum amount of K^+ but lack Ca^{++}, *Paramecium* of P_K type have an optimum amount of Ca^{++} but lack K^+.

Calcium plays a double role towards resistance to nickel salts. On the one hand it acts in an opposite way to potassium to reestablish a higher permeability to Ni^{++} and on the other hand it is an antagonist to Ni^{++}. Following experiments permit to define those two parts more precisely.

Grębecki 1965 and Kuźnicki 1966 have observed that a decalcification of the membrane brings on ciliary reversal or makes the influence of factors causing it easier. Recalcification has an opposite effect. Comparison between sensibility of *Paramecium* of P_K and P_{Ca} type to K^+ permits the same observation. In fact the first

ones decalcified are very sensible to K^+, the second recalcified are not so sensible. So the level of Ca^{++} in the membrane determines the intensity of the action of K^+ on resistance to Ni^{++}. Since K^+ does not act by decalcifying membrane, it means that Ca^{++} keeps it in a state the more steady as its level is higher. Potassium alone is then active force which brings on the change of this state, the intensity of this change depending on the ratio of the levels of K^+ and Ca^{++}. Such an explanation does not contradict the results about ciliary reversal, this reversal taking place when this ratio has reached a certain value which can be obtained either by decalcifying membrane or by overloading it with K ions or with ions having a similary influence to that of K^+.

So membrane oscillates between two states: one of them is stabilized by Ca^{++} and the other unsteady, actively obtained by K^+. Thus at this time, the two ions play only an indirect part in the variation of resistance to Ni, in the ciliary reversal or in all the modifications of membrane attending them. In fact these phenomenons depend on the state of a system of the membrane sensible to Ca^{++} and K^+. The necessity of enzymes, therefore of proteins to permit the penetration of Ni^{++}, leads us to examine if the state of these proteins is the determining element for the permeability to Ni^{++}. Demonstration of such a fact extended to ciliary reversal would permit to establish a relation between this one with the sudden passage of the proteins of the membrane from a steady state to an unsteady state obtained by the influence of K^+

4. Study of the kinetics of penetration of Ni^{++} in *Paramecium* of P_{Ca} and P_K type

The initial speed *Vi* of penetration of Ni^{++} in *Paramecium* of P_{Ca} and P_K type has been computed as it is previously indicated. Results are given in Table 6 and Fig. 4.

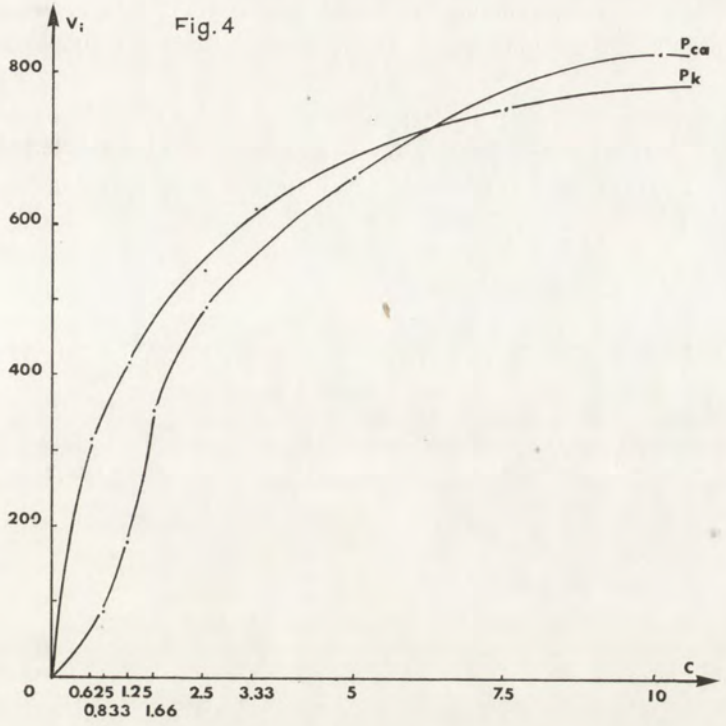
Kinetics of penetration of Ni^{++} in *Paramecium* of P_K type obeys a simple law of Michaelis represented by an hyperbolic curve. In *Paramecium* of P_{Ca} type it obeys

Table 6

Initial speed (*Vi*) of penetration of Ni^{++} in *Paramecium caudatum* of P_{Ca} and P_K type

Concentrations of $NiCl_2\,6H_2O$ in mg/l	0.625	0.833	1.25	1.66	2.5	3.33	5	7.5	10
Vi: *Paramecium* of Pca type	—	84	179	352	490	—	666	—	833
Vi: *Paramecium* of P_K type	312	—	417	—	541	621	—	781	—

a more complex law represented by a sigmoid curve. The passage from P_K type to P_{Ca} type reveals a change in the properties of the enzymatic system. Existence of sigmoid curves perhaps reveals its allosteric nature (Monod et al. 1969). So transition from P_K to P_{Ca} might result from a variation of conformation of proteins used for the penetration of Ni that is from an allosteric transition which might affect them.



5. Study of the influence of Ca^{++} and K^{+} on the kinetics of penetration of Ni^{++}

(a) study in *Paramecium* of P_K type

The initial speed of penetration of Ni^{++} in *Paramecium* of P_K type has been studied in presence of concentration of 25 mg/l of $CaCl_2\ 2H_2O$ and of 50 mg/l of KCl.

Results are given in Table 7 and Fig. 5.

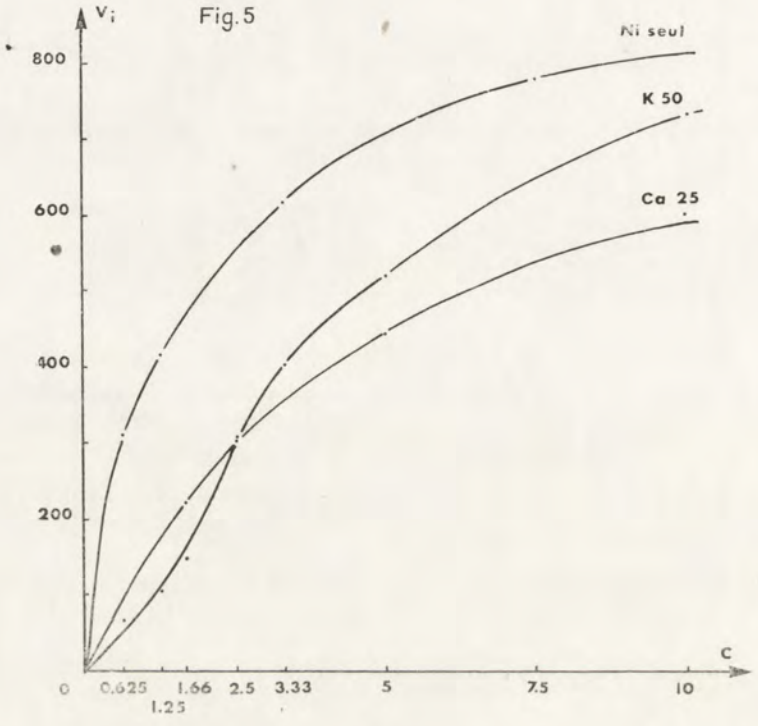
Table 7

Initial speed of penetration of Ni^{++} in *Paramecium caudatum* of P_K type in presence of $CaCl_2\ 2H_2O$ and of KCl

Concentrations of $NiCl_2\ 6H_2O$ in mg/l	0.625	1.25	1.66	2.5	3.33	5	7.5	10
Vi in presence of Ni alone	312	417	—	541	621	—	781	—
Vi in presence of Ni + $CaCl_2\ 2H_2O$ 25 mg/l	—	—	223	305	—	444	—	606
Vi in presence of Ni + KCl 50 mg/l	68	106	148	308	409	523	—	735

Calcium only shows an antagonistic effect against Ni^{++}; characteristics of the kinetics of penetration are unchanged. This kinetics still obeys a simple law of Michaelis.

Potassium, though it reveals an antagonistic effect, makes clear the sigmoid nature of the relation between initial speed and concentration of substrate. It behaves like a negative allosteric effector. It appears to be the active agent bringing on modifications of membrane.



(b) study in *Paramecium* of P_{Ca} type.

The initial speed of penetration of Ni^{++} in *Paramecium* of P_{Ca} type has been studied in presence of concentration of 25 mg/l of $CaCl_2\ 2H_2O$ and a concentration of 100 mg/l of KCl.

Results are given in Table 8 and Fig. 6.

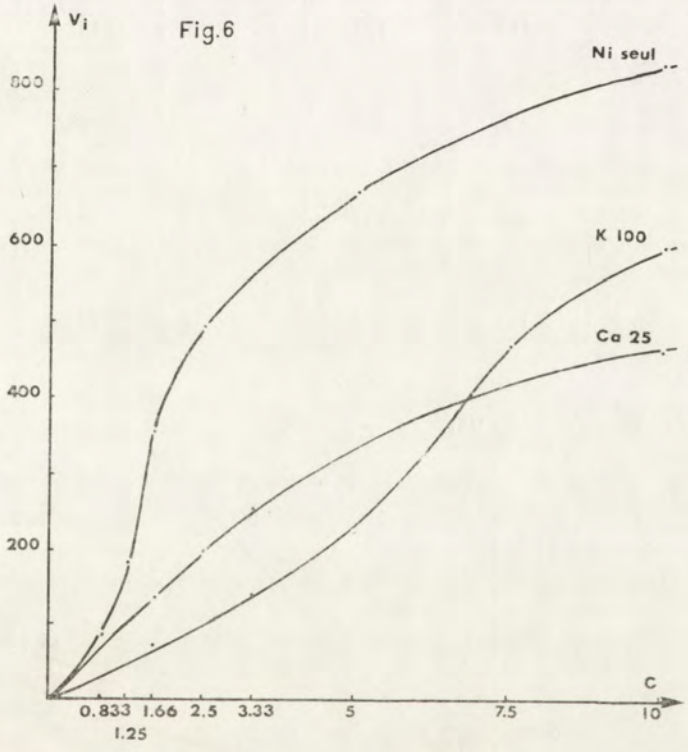
This experiment confirms the nature of negative allosteric effector of potassium which emphasizes the sigmoid form of the curve.

Calcium as we have already noticed plays two parts: on the one hand it is an inhibitor of Ni^{++}, on the other hand it behaves like a positive allosteric effector by restoring the relation of initial speed concentration of substrate to a simple law of Michaelis.

Table 8

Initial speed of penetration of Ni^{++} in *Paramecium caudatum* of P_{Ca} type in presence of $CaCl_2$ $2H_2O$ and KCl

Concentrations of $NiCl_2\,6H_2O$ in mg/l	0.833	1.25	1.66	2.5	3.33	5	7.5	10
Vi in presence of Ni alone	84	179	352	490	—	666	—	833
Vi in presence of Ni + $CaCl_2\,2H_2O$ 25 mg/l	—	—	133	195	250	314	—	459
Vi in presence of Ni + KCl 100 mg/l	—	—	67	78	138	208	463	595



So michaelian kinetics of penetration of Ni in *Paramecium* of P_K type is effectively the result of their overloading with Ca^{++}. Usually positive allosteric effectors are activators of enzymes. This has been observed in the case of *Paramecium* of P_{Ca} type for weak concentrations of Ca^{++}. What is the nature of the antagonism of Ca^{++} which appears then?

6. Study of the inhibition of Ca^{++} on kinetics of penetration of Ni^{++} in *Paramecium* of P_K type

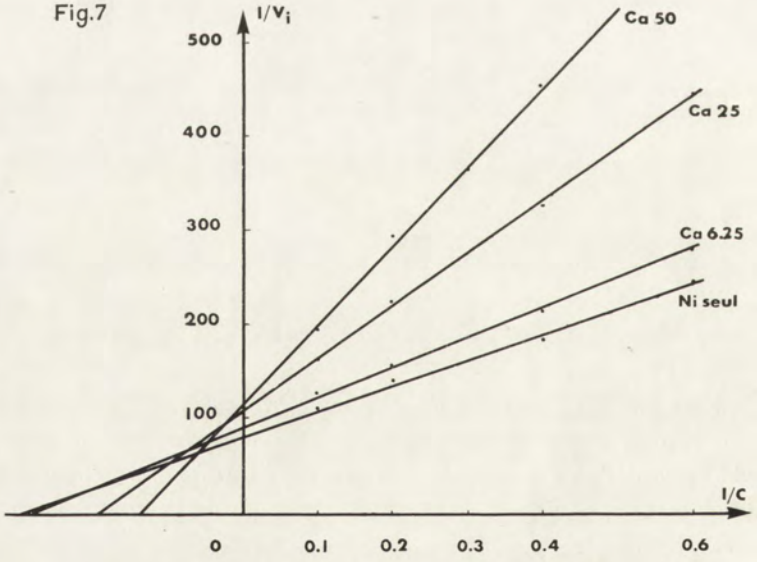
The initial speed of penetration of nickel in *Paramecium* of P_K type has been studied in presence of different concentrations of $CaCl_2$ $2H_2O$. Results are given in Table 9 and Fig. 7 (Lineweaver and Burk plots).

Table 9

Initial speed of penetration of Ni^{++} in *Paramecium caudatum* of P_K type in presence of different concentrations of $CaCl_2\,2H_2O$ (Lineweaver and Burk plots)

$\frac{1}{C_{Ni}}$	0.1	0.2	0.3	0.4	0.6
$\frac{1}{Vi}$ in presence of Ni alone	113	139	—	185	244
$\frac{1}{Vi}$ in presence of $CaCl_2$ 6.25 mg/l	130	160	—	216	279
$\frac{1}{Vi}$ in presence of $CaCl_2$ 25 mg/l	165	225	—	328	448
$\frac{1}{Vi}$ in presence of $CaCl_2$ 50 mg/l	194	294	367	455	—

Fig.7



The inhibition of Ca^{++} towards Ni^{++} appears to be a semi-competitive phenomenon with a very strong tendency to competitivity, tendency which is all the more marked as the concentration of Ca^{++} is stronger. Since the two parts of Ca mix together we can consider that the antagonistic effect is due to a competitive phenomenon with Ni^{++}. Then these two ions might use the same system of penetration into the cell. But since these ions behave very differently the hypothesis needs to be confirmed by other experiments.

Conclusion

The observation of sigmoid curves and of their modifications under the influence of K^+ and Ca^{++} ions brings a proof in favour of the allosteric nature of the enzymatic system permitting penetration of Ni^{++}. Ca^{++} might be a positive allosteric effector, K^+ a negative effector. At the same time Ca^{++} would behave as a competitive ion toward Ni^{++}. According to the ratio between the levels of K^+ and Ca^{++}, this system might oscillate between two states, one of them might be stabilized by Ca^{++}, K^+ being the active agent allowing the allosteric transition of the proteinic protomers from one state to another.

The state of each protomer might depend on the interactions between the binding sites of the three ions.

In spite of some differences we have noticed, numerous ressemblances between the action of K^+ and Ca^{++} ions on resistance to Ni^{++} on the one hand and their action on ciliary reversal our hypothesis could be extended to the explanation of this last one. The state of the proteins of membrane might determine the direction of ciliary beat. This state might be determined by the level of K^+ and Ca^{++} ions.

The existence of such allosteric transition might permit explanation of all or none response, that is of the sudden variations of the properties of membrane in relation with weak variations in stimulus (P.C.R. phenomenon).

The sudden change in the conformation of proteins which can be accompanied by a sudden change in the permeability to ions might explain the concomittance between depolarization of the membrane and ciliary reversal.

Every perturbation, either general or punctual (variation in the amount of ion, variation in the repartition of electric charges under the influence of an electric field, impacts) apt to modify, utterly or locally, the equilibrium state of protein sub-units, because their cooperativity, could bring on a general perturbation of the membrane.

The model of Monod, Wyman and Changeux supposes the association of protomers in oligomers having an axis of symmetry. This implies, as it is noted by Changeux 1968 and Kilkson 1968, the organization of the membrane in sub-units having a polarity and situated in a plane according to a disposition tangentially polarized. The membrane of *Paramecium* as it is noticed by the first author has obvio-

usly such an orientation in the plane. Biophysical studies of the molecular interactions in the membrane and of their variations during ciliary reversal can bring interesting precisions about it, and confirm the hypothesis that we formulate in this work.

Summary

Study of the antagonism of K^+ and Ca^{++} ions towards the resistance to nickel salts shows that both of them act as allosteric effectors of the enzymatic system allowing penetration of Ni^{++} ions. At the same time Ca^{++} might be competitive with Ni^{++}, using the same transporting system. The last one, according to the ratio of K^+ and Ca^{++} levels, might oscillate between two states, the first one being stabilized by Ca^{++} and the second one obtained by an allosteric transition under the action of K^+.

This hypothesis might be extended to ciliary reversal, state of the proteins of membrane determining the level of its polarization and the direction of ciliary beat.

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Spectral activity of light and phototaxis in *Paramecium bursaria*

Paramecium bursaria is a protozoan always occurring with symbiotic algae of the genus *Chlorella*, responsible for its characteristic green colour (Pl. I 1). This protozoan is a classical example of obligatory symbiosis and has been the subject of numerous investigations, mainly on its trophic conditions, growth, culture development and the genetics of its endosymbionts, whereas the question of its phototaxis is still open. Up till now the problem of phototactic sensitivity of *Paramecium bursaria* has not been satisfactorily worked out. The author of this paper has failed to encounter any research warks on the phototaxis of *Paramecium bursaria*, except for a short note by Wichterman (of 1948) stating that phototactically this organism is positively sensitive to white light, whereas all other paramecia do not exhibit this sensitivity.

The protozoans *Paramecium bursaria* taken for the experiments were caught in the field, while the experimental clone was derived from one specimen. The protozoans were cultured in light thermostats under white light of fluorescent tubes with intensity about 1000 lux at 26–27°C. The medium was nutrient solution made from dry lettuce leaves – *Lactuca sativa* – according to Sonneborn's prescription (1950). This mass-breeding was carried out in cuboidal glass vessels 240 mm high with capacity 600 ml exposed continuously to white light. For each experimental series new material was used, derived from condensing the protozoans to a constant density and by further transferring them on a new medium.

The basic apparatus used for the phototactic measurement of the protozoans population is shown in Fig. 1. The cuvette (A) with capacity 250 ml was placed in a light-tight casing (B) provided with two round holes, one of which contained selenium cell (F) connected to a graduated galvanometer (G). This front side of the casing was fitted with a holder for interference filters and neutral nett filters. As a light source served a 1000 W projection filament bulb (Z) connected to a voltage control (T) and an equalizer (S). The recording of changes in the medium's optical density might be effected either continuously by connecting the apparatus to a self-recording instrument or by taking periodical readings from the galvanometer. However, it

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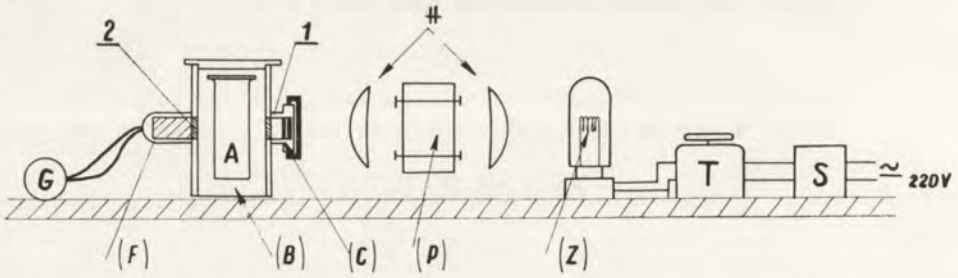

Fig. 1. Scheme of apparatus used for radiation and measurements: A — cuvette with capacity 250 ml, B — light-tight casing, F — selenium cell, G — graduated galvanometer, C — holder for interference filters and neutral nett filters, Z — 1000 W projection filament bulb, T — voltage control, S — equalizer, P — thermical filter with H_2O, H — glass lenses, 1 and 2 round holes (1 containing selenium cell)

appeared more useful to record the phototactic changes at certain intervals of time, e.g. every 5, 10 or 15 min, applying for this purpose a brief pulse of white light with relatively low tension which on its way penetrated through the suspension in the cuvette and fell on the photocell.

The response was counted positive when the protozoans accumulated in the lightened area, gathering on the front wall of the cuvette or while swimming in the light field. Soon began to appear a green "phototactic spot" the intensity of which was gradually increasing till the moment when the process became stabilized.

The pictures of the "phototactic spot" together with the area adjacent to it — the so-called "background" — are shown in Pl. I 2, 3. The difference in the optical density of the "background" as contrasted with that of the "phototactic spot" is markedly apparent. The protozoans taken for the phototactic measurements were first condensed in a mass culture and transferred to a new medium made from dry lettuce leaves of *Lactuca sativa* and distilled water added in proportion 1 : 3. The medium obtained in this way was then kept in dark for 24 h. After this, the cuvette was taken out of the thermostat and placed in the darkening casing of the measuring apparatus, the casing of which was carefully protected against light from the outside. After 1–2 h a measurement on the medium's density was taken, the so-called "background", which was an index of the protozoans' dispersion in the dark for the given series. The measurements contained to be taken every 10 min till three successive readings gave equal results.

The white light intensity was measured in luxes or in quantums/cm^2 sec applying monochromatic light ranging between 375–725 nm. The preliminary measuring results taken during the first 3 h with the application of white light are shown in Fig. 2. It appeared that at a relatively very low light intensity (90 lux), just as in the dark, the protozoans' condensation became somewhat lesser with increasing time due to the sinking of the protozoans on the bottom of the cuvette. At a little higher light intensity (250 lux) the galvanometer indications did not cease; at that intensity the

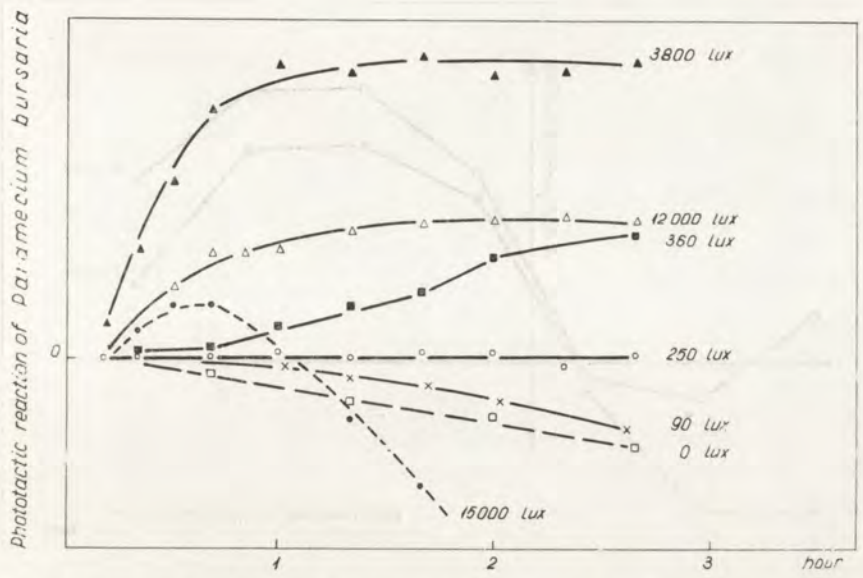

Fig. 2. Influence of different light intensities on the protozoans accumulation: x axis—time in hours, y axis—phototactic reaction measured by optical density of the spot (number by the curves, denote light intensity in lux)

sinking of protozoans was compensated by the phototaxis. Higher intensities, depend ing on their values resulted in a marked accumulation of the protozoans in the ligh field. Very high intensities of the order of 15 000 lux after a previous momentary accumulation resulted in a flight of the protozoans from the light field, which meant the negative phototaxis. The phototactic optimum was found between 3500–4200 lux.

The results obtained for the phototaxis of *Paramecium bursaria* in white light may be compared with those of Nultsch (1962) and Drews (1959) who estimate the photokinetic sensitivity threshold for various purple bacteria and blue-green algae at less than 1 lux, exceptionally only at 20 lux, while for the diatome *Navicula* this value is 50 lux. Similary, the "photokinetic optimum" was estimated for various organisms, e.g. at 2000 lux for *Phormidium*, whereas the negative photokinesis was observed by Nultsch at very high white light intensities, viz. 30 000 lux.

The experiments in chromatic light aimed to obtain the "action spectrum" in the phototaxis of *Paramecium bursaria*; to this purpose chromatic light of 375–725 nm was produced by using interference filters. In order to supply enough chromatic light needed for these experiments some preliminary trials were taken which revealed that the quantum density = 10 000 quantum/cm² sec was the most appropriate because, as it is shown in Fig. 3 (the point is indicated with a vertical arrow on the graph) this value lies markedly beyond the "phototactic optimum" and thus beyond the saturation point, thereby excluding an interference of the negative phototactic reaction.

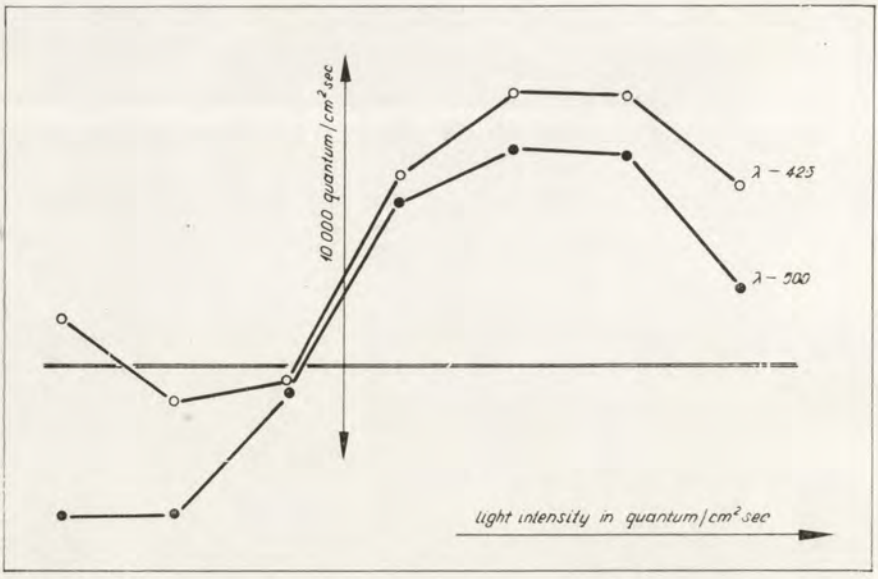

Fig. 3. Influence of radiation intensity with wavelengths 425 and 500 nm on the phototactic effect (above the double horizontal line the reaction is positive; below it is negative)

The author has observed that the phototaxis of *Paramecium bursaria* depends on duration of light stimulus and its wavelength. In view of this two different phases in the phototaxis of *Paramecium bursaria* may be distinguished:

(1) early-appearing phototaxis (the "fast" one) which ensues very quickly and yields measurable results after 2 min (the phototactic optimum may be obtained after 10 min);

(2) delayed phototaxis, during which the whole reaction and the phototactic optimum occur after about 3.5 h.

Figure 4 presents the two aforementioned phases. It should be noticed that the "fast" phototaxis is caused by short wavelengths, with wavelengths 436 and 450 nm as the most active in this process. However, when the phototaxis was examined for a longer time, i.e. 3.5 h, the action spectrum of this process was different (see Fig. 4, the graph below); only after 90 min the effect of red light became visible, the most quickly at 680 nm. This reaction reached its maximum after about 3 h (Fig. 4).

The present paper did not claim to determine the type of the phototactic reaction, i.e. whether in *Paramecium bursaria* it is "topic" or "phobic". Based on numerous earlier observations carried out in our laboratory it may be assumed that the "topic" reaction, i.e. "phototopotaxis" prevails, but nevertheless a simultaneous "phobic" reaction in these protozoans must not be wholly excluded, and hence the "phototactic spots" observed might be likely taken for "light traps", as observed by Engelman in other organisms. This problem, then, will have to be solved later on.

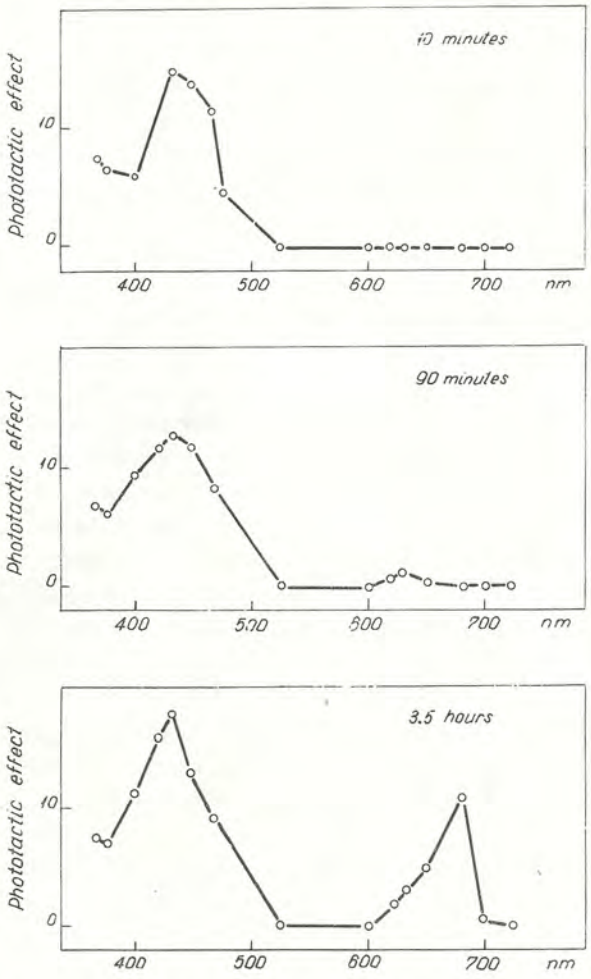

Fig. 4. Effect of radiation spectrum on the concentration of protozoans in the "phototactic spot": x axis—wavelength in nm, y axis—phototactic effect. (Upper curve represents a state after 10 min, the middle one after 90 min, the lower after 3.5 h)

Meanwhile, by a cursory analysis of the "action spectrum" obtained in the phototaxis of *Paramecium bursaria* (see Fig. 4), two distinct phases of the said process may be easily distinguished, what has been already said:

(1) early appearing phototaxis, and

(2) delayed phototaxis.

In view of this two distinct systems controlling the phototaxis may be distinguished:

(1) early-appearing system operating within the range of 375–475 nm (with a maximum at about 450 nm), suggesting the role of riboflavin or carotenoids as

a photoreceptor; this system operates very quickly and is probably very sensitive. Its task may be to bring quickly the symbiotic infusorian into the light field, i.e. the area where green vegetable endosymbionts may initiate their photosynthetic production.

(2) a long-time system which occurs much later and those action spectrum may be identified with the chlorophyll absorption spectrum. This system may be called "photosynthetic".

The information obtained here may not be easily compared with other results regarding the phototaxis of other animal organisms co-existing with endosymbiotic algae on account of the lack of such data in the literature. A comparison with the photoaxis of photosynthetic bacteria as well as with the moving algae leads to a conclusion that in those organisms the function of a photoreceptor controlling the photokinetic or the phototactic reaction is performed by photosynthetic pigments. This has been already pointed out by Nultsch for *Phormidium uncinatum*, by Throm for photosynthesizing bacteria and by Virgin for numerous other algae. On the other hand, the flavin system has been found to control the locomotive reactions (in case of vegetal phototropism) and the chloroplast displacement (Haupt und Schoenfeld 1962, Zurzycki, 1962, 1967). Only exceptionally in some objects (*Elodea*, *Vallisneria*) and additional, although secondary effect of the long-wave component (the photosynthetic one) on the control of photodinesis and chloroplast displacement has been noticed (see Seitz 1964, 1967).

The joint action of the short-wave and photosynthetic systems also occurs in such a highly specialized, in respect of phototactic reaction, organism as *Euglena* (Wolken and Shin 1968) and is also suggested for the phototactic motion of algae (Nultsch 1970).

The still up-to-date hypotheses of the symbiotic origin of chloroplasts due to evolutional transformations of endosymbiotic blue-green algae admit a presumption that the behaviour of protozoans containing endosymbiotic algae will give valuable information on the phototaxis of both the algae and the chloroplasts.

The results presented here may serve as a report from this field, while the mechanism of phototactic responses in *Paramecium bursaria* will be a subject for further investigations.

Acknowledgement

I wish to express my gratitude to prof. dr. J. Zurzycki for entrusting me with elaboration of such an interesting problem and for his valuable advice and interest taken in the course of experiments.

Summary

The problem of positive phototactic responses of the whole protozoans' population *Paramecium bursaria* has been presented in this paper.

The response was counted positive then the protozoans accumulated in the lightened area,

gathering on the front wall of the cuvette or while swimming in the light field, forming then characteristic green "phototactic spots".

Intensities higher than 250 lux, depending on their values, resulted in a marked accumulation of the protozoans in the light field. The phototactic optimum was found between 3500–4200 lux.

The experiments in chromatic light aimed at obtaining the "action spectrum" in the phototaxis of *Paramecium bursaria*; to this purpose chromatic light of 375–725 nm was produced by using interference filters.

The author has observed that the phototaxis of *Paramecium bursaria* depends on duration of light stimulus and its wavelength. In view of this two different phases in the phototaxis of *Paramecium bursaria* may be distinguished:

(1) early-appearing phototaxis. The "fast" phototaxis is caused by short wavelengths, with wavelengths 436 and 450 nm as the most active in this process. This suggests the role of riboflavin or carotenoids as photoreceptor; this system operates very quickly and is probably very sensitive;

(2) delayed phototaxis, which occurs much later and whose action spectrum may be identified with the chlorophyll absorption spectrum. This system may be called "photosynthetic".

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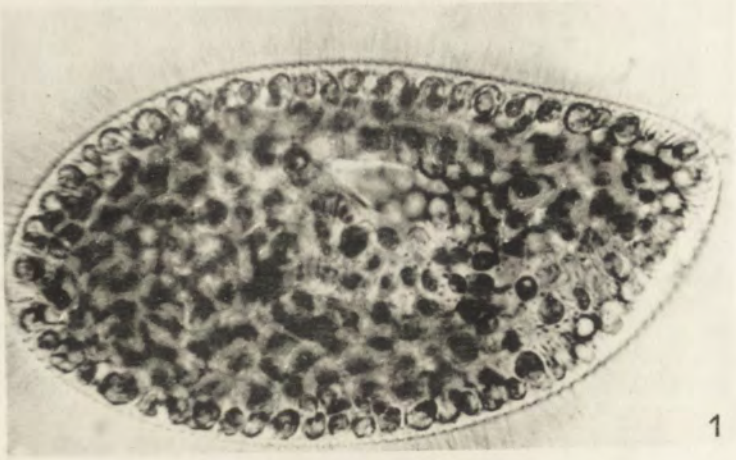
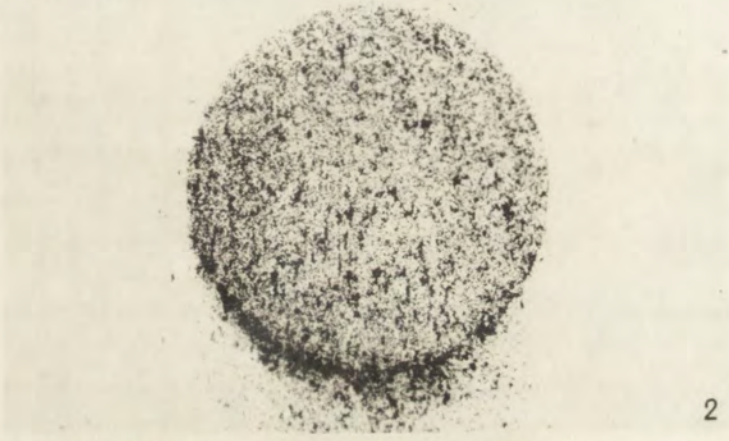
EXPLANATION OF PLATE I

1: Microscopic picture of one specimen of *Paramecium bursaria*
2: "Phototactic spot" (diameter of illuminated field = 22 mm)
3: Part of "phototactic spot" magnified





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Contraction and relaxation of *Stentor coeruleus* in response to mechanical and chemical stimuli

The response of *Stentor coeruleus* to mechanical and chemical stimulation was subject of many earlier studies. It is particularly introduced in Tartar's monograph (1961) "The Biology of *Stentor*". Recently Dryl (1969) brought evidence, that *Stentor coeruleus* after 30 min exposure to medium containing high concentration of potassium ions in presence of calcium (12–16 mM KCl+1 mM $CaCl_2$+1 mM Tris-HCl buffer solution of pH=7.2) becomes insensitive to mechanical and chemical stimulation, although the myonemes are still responding with contraction when stimulated directly by intracellular insertion of microneedle. He suggested, that K^+/Ca^{++} factor inhibits important, physiological mechanism within cell membrane responsible for conduction of impulse from the cell surface to myonemes apparatus located under the pellicle. The present study is an additional attempt to verify Dryl's hypothesis in comparative studies on response of *Stentor* to external stimuli at various conditions of excitability, due to short or longer duration of acting stimulus.

Material and methods

Experiments were carried out with animals kept for some time in 1 mM $CaCl_2$+1 mM Tris-HCl buffer of pH 7.3, which constituted also the control medium.

The procedure of experiment was following: Before starting the experimental series the animals were transfered from nutrient to Ca-Tris-buffer solution for one to two hours. Then, a part of them was put for 30 min into solutions:

(A) 1 mM $CaCl_2$+1 mM Tris-HCl buffer solution without potassium, which served as control medium.

(B) 8 mM KCl+1 mM $CaCl_2$+1 mM Tris-HCl buffer solution.

(C) 16 mM KCl+1 mM $CaCl_2$+1 mM Tris-HCl buffer solution.

In order to control the reaction of animals to external factors, stentors were exposed in small vessels to mechanical stimulation by magnetic mixer moving at constant speed or were stimulated by insertion of microneedle into the interior of cell, while in the case of chemical stimulation the animals well exposed to solution A, B or C.

The response of animals to mechanical stimuli was checked every two minutes.

Paper presented at Symposium "Motile Systems of Cells", Kraków, August 3–7, 1971.

Magnetic mixer was a continuous acting stimulus throught 30 min. The mixer was 2 cm long and had the speed of rotation 14 times per second. The calculated mean rate of liquid flow in the vessel was 80–90 cm/sec approximately. The observations were done at low magnification microscope on 30–40 animals exposed to experimental solution. The first observations revelated that already during first seconds of acting mechanical stimulus 100% of animals show the maximum of contraction of body in the form of gently rounded cone.

The impaling with microneedle was a single short-lasting mechanical stimulus. When the maximum extended animals were impaled, they took immediately the form of gently rounded cone. Observations persisted until the animal body was again completely extended to tube form. The experiments were carried out at temperature 22–24°C.

Results

The preliminary observations revealed that stentors exposed to solution A have a form of extended tube (Plate I 1) or so-called "half-tube" (Plate I 2) which seems to be very characteristic shape of body for stentors swimming freely in the liquid medium. The short-lasting mechanical stimulation by water shake, rapid pippeting etc. causes almost immediate contraction of body in the form of gently rounded cone (Plate I 3) which is followed by slow, gradual relaxation process til the animals after 60–90 sec will recover again the half-tube or extended tube form.

Stentors exposed to solution B or C show during first minutes strong contraction of the body which is accompanied by continuous reversal of beat of AZM and body cilia. In solution B the animals are completely relaxed after 2–5 min while in solution C – after 5–9 min. After 30 min of exposure in the experimental medium the animals recover typical shape of extended tube and show inhibition of movement within membranelles of AZM and very slow reversed beat of body cilia. Under these conditions both groups of animals show no contraction of body in response to short-lasting mechanical stimulation.

The effects of continuous mechanical stimulation on *Stentor coeruleus* exposed previously for 30 min to solution A, B and C, are graphically presented on Fig. 1 which shows the percentage of contracted animals in dependence on the duration of stimulation in minutes.

Each point on the curves represents percentage of animals contracted in relation to all specimens calculated from 10 series of experiments.

Before the beginning of stimulation in solution A all animals were not completely extended, showing a half-tube form. After the first two minutes of stimulation, stentors were contracted, taking the form of gently rounded cone. Later on, the number of contracted animals decreased gradually. The first half tube forms appeared again after 16 min of stimulation. Their number after 20 min achieved above 50%.

In solution B, at the beginning of experiment *Stentor* had the extended tube form but 2 min later all animals were contracted, taking the shape of gently rounded

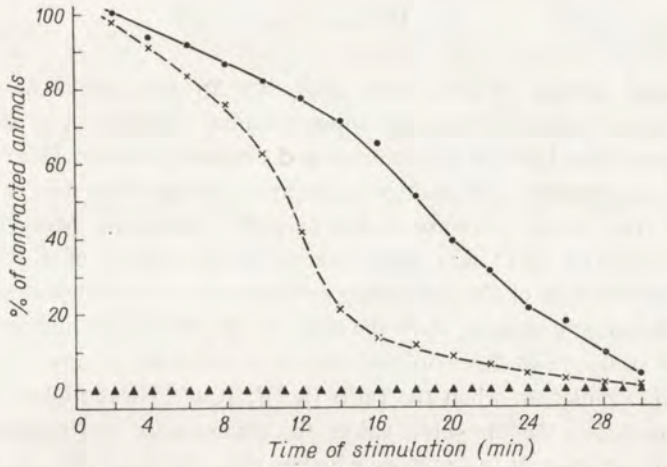

Fig. 1. Response of *Stentor coeruleus* to continuous mechanical stimulation. ——Tris-HCl + 1 mM $CaCl_2$ pH 7.3 (A solution A) —— 8 mM KCl (solution B), ▲▲▲▲ 16 mM KCl (mean value and standard deviation (solution C). A statistical comparison of two cumulative distributions (concerning the data representing the mean arithmetical values of contracted animals in solutions A and B (calculated by the two-tailed Kolmogorov–Smirnov test showed that they differ statistically in significant way (p. 0.001)

cone. Between 2nd and 8th min of exposure the first early stages of *Stentor* extension were observed. In the eight minute appeared the half-tube forms, while, during 12th min these forms achieved more than 50%.

In solution C the animals didn't show any contraction response to continuous mechanical stimulation, and this inhibition of response was characteristic for animals exposed to high concentrations of potassium ions in external medium.

It was proved that animals from all three groups responded with contraction of body when the contractile apparatus within the cell interior was stimulated directly by insertion of the microneedle. The complete extension of the body after impaling of the microneedle was observed between 60–90 sec in animals exposed to solution C and between 20–35 sec in animals from control solution A.

The achieved results brought evidence that exposure of *Stentor coeruleus* for longer period of time to strong continuous mechanical stimulus causes more than 18 min lasting contraction of body which is followed by gradual relaxation so that after 25–30 min of stimulation 100 per cent of animals are relaxed.

Animals exposed to solution B still responded to continuous mechanical stimulation with contraction but they reached relaxation stage much earlier than control animals. However, animals exposed to solution C did not show any contraction response to continuous mechanical stimulation with magnetic mixer, although the myonemes apparatus located under pellicle was physiologically intact as it was evident from its quick contraction in response to the direct stimulation by insertion of microneedle into the cell interior.

Discussion

It was stated already by Jennings 1904 that *Stentor* may show a number of motor reactions of adaptive character when stimulated with carmin particles which can act on peristome both as mechanical and chemical stimuli. Dierks 1926 was able to show that stentors exposed to potassium reach medium may show complete inhibition of contraction response to mechanical stimulation. More recently Pietrowicz-Kosmynka 1972 was analyzing inhibiting effects of K^+/Ca^{++} factor on chemotactic response of *Stentor*, giving evidence that the animals cease to respond to negative chemotactic stimuli when the ratio $[K^+]/\sqrt{[Ca^{++}]}$ is higher than 1.5–2.0. In the present study the authors noticed complete inhibition of contraction response to mechanical stimulation when the ratio $[K^+]/\sqrt{[Ca^{++}]}$ was higher than 8.0 and this may suggest that the threshold values for chemotactic and mechanical stimuli differ in significant way. It is not clear whether this difference depends on existence of various receptors for both kinds of stimuli or is connected with various ways of conduction of stimulus from the cell surface to corresponding effectors, i.e., to cilia and or to myonemes. In any case the achieved results in this study support the view of Dryl that potassium and calcium ions at appropriate concentration in external medium can block the conduction of stimuli from cell surface to contractile apparatus located under the pellicle.

The gradual decline of contraction response in *Stentor* to continuous mechanical stimulation reminds of similar findings reported by Wawrzyńczyk 1937 and Kinastowski 1963 for *Spirostomum* in response to water shake. Wawrzyńczyk explained the phenomenon in terms of learning, while Kinastowski was not able to exclude the occurrence of a kind of "fatigue" which could depend on some cytopathological changes within pellicle or locomotor apparatus.

The present data exclude occurrence of any serious damage to the cell caused by applied mechanical stimulus since it was proved that after cessation of stimulation stentors recover rather promptly (after 1–2 min) the ability to react again with contraction to mechanical stimulus. It seems therefore that the phenomenon can be explained in terms of habituation.

Summary

Stentors exposed for 30 min to potassium reach medium (8 mM KCl+1 mM $CaCl_2$ or 16 mM KCl+1 mM $CaCl_2$) show inhibition of the activity of AZM, reversal of beat of somatic cilia and complete relaxation of body. When under these conditions stentors are exposed to continuous mechanical stimulation (magnetic mixer causing constant rotation of fluid at average rate of 50 cm/sec), the animals kept in 8 mM KCl showed contraction response lasting shorter period of time than in controls while the animals exposed to 16 mM KCl showed no contraction response at all, although it was proved by direct stimulation of cell interior with the microneedle that the myonemes apparatus was still contractile.

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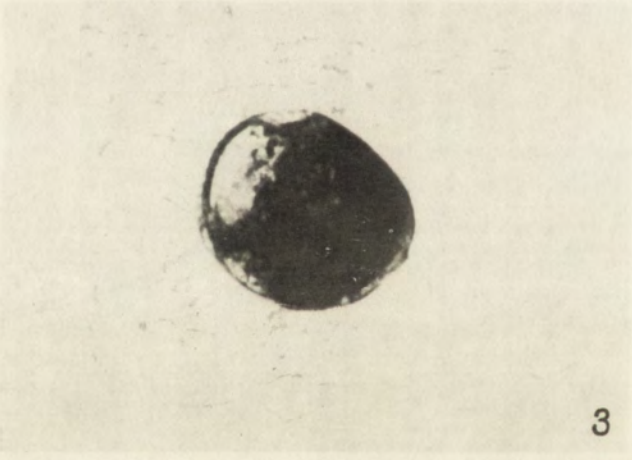
EXPLANATION OF PLATE I

The shape of *Stentor coeruleus* at various stages of contraction
1: Full extension (tube form)
2: Half extension (half tube form)
3: Full contraction (gently rounded cone form) × 75



M. Łukowicz et M. Morawka auctores phot.

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Galvanotactic response and food vacuole formation in *Tetrahymena pyriformis*

The food vacuole formation in *Protozoa* is a process in which the cell membrane flow, vesiculation and permeability plays essential role. Experimental studies connected with phagocytic activity in changing environmental conditions were carried out on various species of *Protozoa*. In the ciliate the important question arises about possible relation between changes of ciliary movement and the rate of suspension intake. The earlier studies (Dryl, Brutkowska and Sikora 1963, Brutkowska 1963, 1967 a, b) showed that there is no direct relation between kinetic and phagocytic effect of those external factors, which change excitability state of the cell membrane. Recent observations on *Tetrahymena* revealed similar relations (Brutkowska unpublished).

In connection with this it seemed to be attractive to make observations on food vacuole formation in animals exposed for some time to direct current which causes depolarization of the cell membrane on cathodal and hyperpolarization on anodal pole. The first proofs on *Paramecium caudatum* (Dryl and Brutkowska 1963) brought evidence that the strong direct current inhibited phygocytosis, while the weaker currents could even stimulate the formation of food vacuoles.

The aim of the present study was to elucidate the role of direct current action on phagocytosis in *Tetrahymena* in dependence on changing composition of external medium.

Material and methods

Tetrahymena pyriformis, strain G. L. was cultivated on 2% proteose-peptone (Difco) solution and was washed exactly with 1 mM Tris-HCl sol. at pH 6.9 without Ca^{++} ions by using hand centrifuge at least 1 h before experiment. The animals kept in this control medium without addition of other ions were called "control animals". Their phagocytic activity was controled at first. The experiments were carried out at temperature 22–24°C.

The procedure of experiments was following: one part of carefully prepared carmin suspension was mixed with one part of distilled water in the case of control proof, or with one part

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of appropriate amount of KCl, NaCl, $CaCl_2$, $MgCl_2$ and Tris without Ca^{++} to get the final concentration of 20 mM in each case, and so prepared medium was mixed with two parts of "control" animals. After 10 min of staying in these conditions animals were immobilized by 10 mM $NiSO_4$ and the number of carmin filled food vacuoles was counted in 20 specimens. In experiments with exposition to d.c., the animals were at the beginning exposed for 1 min to acting current in medium devoid of carmin suspension, which was added thereafter. And they were finally exposed for 10 min to d.c. containing carmin suspension with one of the above mentioned chemical factors in 20 mM concentration.

The exposure to d.c. was carried out in the small glass chamber, approximately 7.5 cm long, 0.5 cm wide and 1 mm deep. with electrodes located at distal ends.

The threshold value of galvanotactic response was corresponding to 70–80% of animals swimming towards cathode.

Results and discussion

The behavior of animals exposed to direct current

Tetrahymena shows the cathodal galvanotaxis when exposed to d.c. and similarly as in *Paramecium* and many other ciliates this orientation is caused by reversal of ciliary beat on the cathodal side of the body. However, it should be pointed out that animals attached to the substrate show the cathodal oblique response with the anterior end of the body pointing out NE–SW orientation when the cathode is on the left and when looking downwards to the substrate. This oblique thigmogalvanotactic response is due to the assymetry of strength of ciliary beat on both sides of thigmotactic animal. As a rule thigmotactic specimens are attached to the substrate with the left margin of the body and with the peristome on the right (Fig. 1).

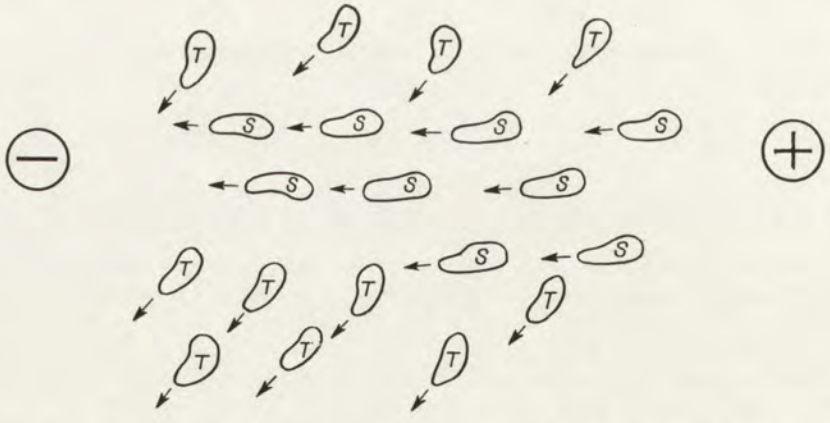

Fig. 1. Galvanotactic response of *Tetrahymena*. S—swimming animals showing typical cathodal galvanotaxis E–W, T—thigmotactic animals showing oblique galvanotaxis NE–SW, ⊖—cathode, ⊕—anode

Table 1

The rate of food vacuole formation in *Tetrahymena pyriformis* during direct current stimulation in various media

Applied voltage		Composition of external medium					
		1 mM Tris HCl	20 mM KCl +1 mM Tris HCl	20 mM NaCl +1 mM Tris HCl	20 mM $CaCl_2$ +1 mM Tris HCl	20 mM $MgCl_2$ +1 mM Tris HCl	20 mM Tris HCl
26.0 V/cm	A	2.5±0.94	0	0	0	0	–
12.0 V/cm	A	2.5±0.50	3.6±0.89	0	0	0	–
	B	2.8±1.19	3.2±0.87	1.2±0.56	0	0	1.9±0.68
6.0 V/cm	A	3.7±1.52	3.3±1.55	2.2±0.64	1.6±0.86	0	–
	B	3.0±0.74	2.2±0.90	1.7±0.63	0	0	2.0±0.00
Control	A	3.4±0.66	3.7±0.54	3.1±0.70	3.8±1.92	3.3±1.15	–
	B	4.2±0.46	3.1±2.26	3.2±0.75	3.7±1.75	3.0±1.71	4.3±1.60

A, B – Two separate series of experiments.

0 – No food vacuoles formed.

The rate of f.v. formation was calculated as a mean number of food vacuoles formed by 20 animals during 10 min of exposure to suspension of carmin particles.

Phagocytic activity during exposure to direct current

It is shown on the Table 1 that direct current inhibits phagocytic activity of *Tetrahymena*. As a rule the number of food vacuoles is lower when the voltage of current increases. This inhibiting process is concerned also with changes in size and shape of food vacuoles and with intensity of filling of vacuoles with carmin suspension. In some cases a part of animals doesn't form food vacuoles. Since the number of animals devoid of food vacuoles is increasing paralell to higher voltage, it is suggested that phygocytic activity of these organisms is completely inhibited.

Another interesting problem, which appeared during these experiments is connected with joint effect both of direct current and cations on *Tetrahymena* phygocytosis. It should be pointed out that even high concentration of chloride solutions didn't much decrease the number of suspension filled food vacuoles when aplied alone i.e., without d.c. stimulation. The same was true for experiments carried out with animals exposed to d.c., but without addition of salt solutions. However, the joint action of these two agents resulted in higher degree of the inhibition of food vacuoles formation.

This synergic effect of d.c. on food vacuoles formation was more expressed in the media containing divalent cations. Among them the number of food vacuoles was more decreased in the presence of 20 mM $MgCl_2$ solution, than of 20 mM $CaCl_2$ solution. Among monovalent cations in the case of 20 mM soln. of KCl and simultaneous stimulation the number of food vacuoles was relatively stable, whereas it was decreased in the presence of 20 mM NaCl solution. D.c. was also acting strongly in 20 mM Tris-HCl sol. of pH=7.2.

On the other hand, however, it was found that the cations used in these experiments didn't effect much the galvanotactic thresholds, the values of which were 1.1–1.3 V/cm in control and monovalent cations media, and 1.6–1.7 V/cm, in media containing bivalent cations.

Since inhibition of food vacuoles formation in the presence of cations occurs at much higher voltage of applied d.c. than the threshold value for galvanotaxis, the authors put in doubt possibility of any direct relation between effects of d.c. stimulation on the cell excitability and phygocytosis in *Tetrahymena*.

Brandt and Freeman 1967 found antagonistic effects of calcium and sodium ions on the functions of cell membrane in amoeba; pinocytosis appeared at higher level of sodium ions in external medium while it was inhibited by increased concentration of calcium ions. Inhibiting effects of calcium on food vacuoles formation were reported recently by Nilsson 1971 and this may suggest that both phenomena (i.e., pinocytosis and phygocytosis) are negatively affected by high concentrations of external calcium. The results presented in this paper suggested that magnesium ions are even more active in this respect. Recently Bhownick 1970 demonstrated that some structural changes of mitochondria of *Tetrahymena* could be induced

by repeated electric shocks of d.c. but so far it is rather difficult to conclude whether these changes can affect the course of phagocytosis although such possibility can not be ruled out.

Summary

The inhibition of food vacuole formation rate in *Tetrahymena pyriformis* occurs at much higher values of applied d.c. stimulation (12.0 V/cm) than the threshold level for galvanotaxis (1.3–1.7 V/cm).

High concentrations of cations (20 mM concentrations of NaCl, KCl, $CaCl_2$) had little effect on the rate of food vacuole formation, while the simultaneous exposure of animals to d.c. caused inhibition of phagocytic activity. The synergic effect of d.c. and cations on the process of phagocytosis was specially strongly marked in the case of divalent cations Ca^{++} and Mg^{++}

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A note on changes in the movement of *Colpoda* in relation to treatment with actinomycin

A preliminary analysis of prefission morphogenesis in the Ciliate *Colpoda cucullus* has been done by use of actinomycin D (Crippa Franceschi and Milani 1971). Two hours exposure to 200 μg of the substance per 1.0 ml of culture medium at some given time of the interfission period results in some alterations of cortical and nuclear components in the daughter cells and in following generations.

Before and after a definite interval of the interfission period actinomycin has no effect, and the normal morphogenetical events take place, suggesting that the genes are active or not active at different stages of the cell cycle (Gill and Hanson 1968).

In the course of this research, some observations were occasionally done in relation to the movement of the cells coming from the treated ones. A kind of a conspicuous irregularity seemed to be characteristically present after treatment at 5 1/2 h in comparison with the controls.

These facts were thought to be of interest in relation to the specific effect of actinomycin. Five experiments were done in order to describe the phenomenon and to analyze its relationship to the cortical organization.

Each experiment concerned with: treatment by actinomycin for two hours at the interfission times of 2 1/2, 4 1/2, 5 1/2, 6 1/2 h; replacement of the treated cells into the standard medium; analysis of the movement in the following generations, until the fourth one. The obtained results appeared to be in perfect agreement each to other.

As for the basic knowledge concerning the movement in *Colpoda* the first observations were reported by Franceschi (1958) with regard to some research on "Dauermodifications". More detailed studies on the subject are now in development in Genova by Milani and others. In a general way, it is possible to point out some facts. As in other Ciliates, there is the presence of forward left spiralling (FLS) and forward right spiralling (FRS) movements (Dryl and Grębecki 1966, Grębecki, Kuźnicki and Mikołajczyk 1967, Kuźnicki 1970); the frequency of FRS is extremely low. A very high sensitivity to the stimuli points out some similarities with the case of

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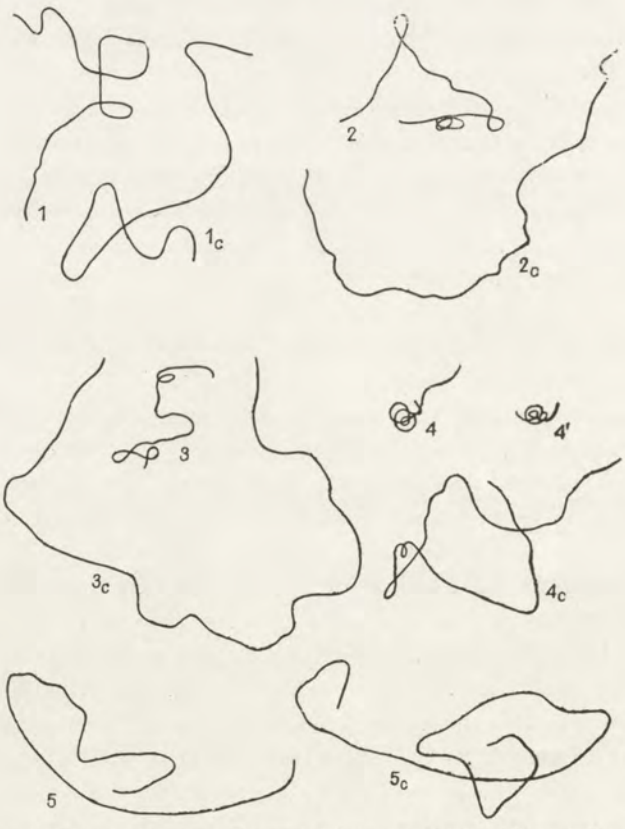
Ophryoglena (Párducz 1964) where it was observed a large possibility of "partial and direct modifications of the ciliary work when the cells are put in front to some difficulties".

The present investigation has been founded above all on two methods, chosen among those available for the study of Ciliate movement. The pathways of the control cells and of the cells coming from treatment by actinomycin, have been studied by camera lucida registrations (15 sec observations), and by the photographic method of Dryl 1958 (2–3 sec exposure time).

The cells were all chosen at the same interfission age, put in a definite medium, and finally studied by single samples in the case of the camera lucida, by groups of animals in the case of the photographic registrations, in the course of some generations (from the first to the fourth one).

Figures 1–5 concern samples taken at random from those studied by camera lucida; a comparison is possible between the first generation of cells coming from



Figs. 1–5. Camera lucida registrations (15 sec) of the pathways followed by *Colpoda cucullus* in the first generation of cells after exposure to actinomycin, in comparison with the controls (c). Treatment applied at various times in the cell cycle: 1—21/2h, 2—41/2h, 3, 4 and 4′—51/2h, 5—61/2h

the treated animals, and controls. The samples coming from the cells treated at 2 1/2h of the interfission period (Fig. 1) show very small differences in comparison with the controls, as regards the shape and the speed of movement. Such differences became strongly marked in the case of 4 1/2 h (Fig. 2), and reached the highest values in the case of 5 1/2 h (Figs. 3, 4), becoming extremely weak in the case of 6 1/2 h of interfission period treatment (Fig. 5).

Analogous results came from the photographic method by which the animals have been followed for a very short time (2–3 sec exposure). As Fig. 6 particularly

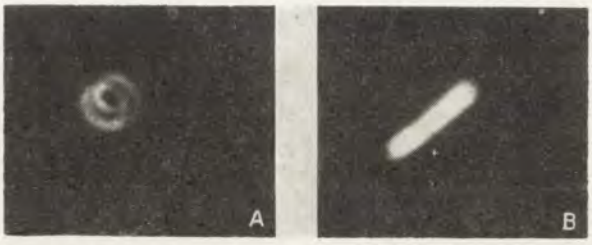


Fig. 6. Photographic method by Dryl (2–3 sec expos.) applied on *Colpoda cucullus*, tracing obtained for: A—first generation of cells treated by actinomycin at 5 1/2 h of the interfission period, B—control

shows, the pathways followed by the animals freely swimming in the liquid and coming from material treated at 5 1/2 h of the interfission period, are expressed by a kind of spiral with a vertical axis which represents the most frequent pattern of alteration of movement when the controls are characterized by a straight regular line.

Together with the data previously described, it must be added that the numerous observations clearly revealed another general characteristic of the cells coming from the ones previously treated. The frequency of FLS, in fact, became lower, and a high frequency of FRS became the characteristic pattern of movement in comparison with the controls, particularly in the case of treatment at 5 1/2 h of the interfission period.

It seemed therefore possible to conclude about some changes in the movement of *Colpoda* strictly depending on actinomycin. Such drug effect could be directly or indirectly effective on the elements of the cell which are in some way responsible for movement (Ehret and Powers 1959, Pitelka 1963, 1969, Nanney 1966, 1967, Heckmann and Frankel 1968, Grain 1969, Hufnagel 1969).

It seemed then possible to find a relationship between the changes in the movement and the alterations of the cortex which had been observed in the course of the analysis of prefission morphogenesis.

A large quantity of the animals showing different degrees of stronger alterations in their movements were stained by the silver impregnation technique suggested by Klein (after Burt 1940). The results showed good agreement with those already found during the research on prefission morphogenesis. The kineties showed different patterns in the cells coming from the treated ones in comparison with the controls,

and the differences noted were strictly depending on the time of the interfission period at which the treatment has been applied.

Figure 7 and Pl. I present the general picture found in a definite portion of the cortical organization in the first generation of the cells after treatment and later, in comparison with the controls. Both camera lucida drawings and photos put in evidence the altered pattern of the kineties in the region 1–7.

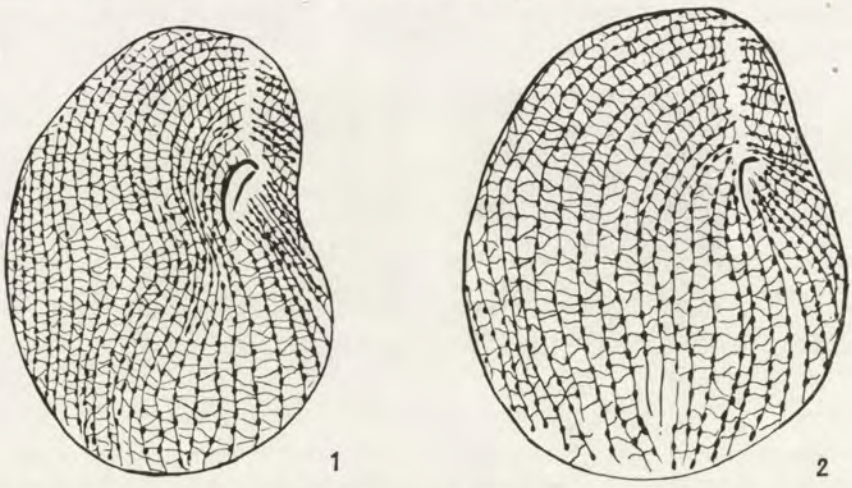


Fig. 7. *Colpoda cucullus*, the cortical organization. A—atypical pattern of the kineties, first generation of cells after exposure to actinomycin at 5 1/2 h of the interfission period, B—control, normal pattern of the kineties

The results obtained lead to the following general conclusions:

There is a strict relationship between actinomycin treatment, changes in the movement of the cells coming from the treated ones, and changes which characterize the cortical organization of the cell. It would be very interesting to postulate a blockage of protein synthesis responsible for some structures of the kineties which could bring a change in the motile system of the cell (Beisson and Sonneborn 1965, Kennedy and Zimmermann 1970, Sonneborn 1970).

However, we do not know enough at the moment about the changes of the pattern of the kineties, whether they are induced directly by the action of the substance, or they appear indirectly as a consequence of some other changes specifically brought by the substance. It is believed that further experiments will bring more information on the subject.

Summary

In the course of a preliminary research on prefission morphogenesis conducted on *Colpoda cucullus* by use of actinomycin D, some alterations in movement have been observed in the first generation of cells after treatment, and in further generations of cells. A high frequency of forward

right spiralling movement, together with some conspicuous variations in the pattern of locomotion and some modifications of speed, are strictly related to the interfission period at which the treatment has been done. The study of the cortical organization of the same cells leads to the conclusion that there is a strict relationship between actinomycin treatment, changes in movement and cortical changes in the cells coming from the treated ones.

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EXPLANATION OF PLATE I

Colpoda cucullus. The cortical organization
1: Atypical pattern of the kineties, first generation of cells after treatment by actinomycin a 5 1/2 h of the interfission period
2: Control, normal pattern of the kineties

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Photoinduction reactions of *Tetrahymena pyriformis* GL

In one of previous reports (Biczók 1969) the author described what kind of influence has been exerted on the photoinduced movement, respiration of *Tetrahymena pyriformis* GL by some photodynamic dyes and various inhibitors (KCN, NaF, *2,4*-dinitrophenol). We were looking for a connection between the intensity of O_2 consumption and the speed of movement as well as the change in electronmicroscopic structure arising out of the photodynamic laesion. We endeavoured to approach the comprehension of the complicated mechanism of photoinduced movement. That is also the aim of the present study.

The author was attracted by the curve of double peaks with two maximums that reflect truly all the external and internal effects influencing directly or indirectly the speed of movement of *Tetrahymena pyriformis*. This was the case of action of the very efficient sensitor of proved value, Rose bengale (Fluka, colour index 45, 435), that diminishes with a dramatic speed the viability of *Ciliata*, shortening the curve of the intensified demonstrative speed of movement, particularly if animals are irradiated by the light of wavelength 5430 Å from a high-pressure mercury vapour lamp or xenon lamp, in compliance to its absorption maximum. As a result of that, also the claim for oxygen increases somewhat, the plasmatic structure changes and – what is the most obvious and momentarily perhaps the most important – the cristae of mitochondria do apparently perish. Of all this has followed, of course, the next problem: how much are these phenomena influenced by the most important energy-supplier of movement, the ATP, and by cysteine that somewhat increases the claim for oxygen and protects visibly against the photo-lesion? In addition, the problem is also raised to follow with attention the intercurrent effect of temperature, taking into consideration, that the temperature coefficient (Q_{10}) is but a little greater than 1.0 concerning the photochemical and photobiological reactions, showing that the speed of the photochemical reactions is influenced only a little by heat.

For the investigations we have used animals bred axenically in darkness, kept for several hours in an defined ion milieu set by phosphate buffer to pH 6.9 (Biczók 1961). The photoinduced O_2-consumption of the adapted animals was measured by a Warburg-apparatus built in by F-tubes of 1105 erg cm^{-2} sec^{-1} intensity. The

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photoinduced speed of the movement was investigated in a hanging-drop with a Zeiss microscope mounted on Stanko's monochromator. The photo-lesion was provoked by an electric bulb of 100 watt from a distance of 40 cm, after filtering out the heat rays in a proper way. We have irradiated the *Tetrahymenae pyriformes* of the single samples until the accelerated movement ceased entirely to exist.

For studying the submicroscopic structure, we have embedded the material, fixed in 1 per cent OsO_4 and than dehydrated, into araldit. As a contrast material, uranylacetate, resp. lead-citrate was used (Biczók and Pardutz 1970).

The ascertainment of the molar concentration of ATP and cysteine of the agent was problematic. The animals could namely often not tolerate the concentrations of 5×10^{-3} M and perished before the end of the experiment. Therefore we kept them using the concentrated solutions 5×10^{-4} M newly prepared before every investigation and added to the experimental object.

For recording and evaluating the results, we have to start from the curve of the movement speed brought about as a result of the light of wave-length 5430 Å of the high-pressure mercury vapour lamp of 500 watt. The curve of movement speed described earlier, arched mildly and expressing the movement of unstained *Tetrahymenae* with its less perceptible double maximum proved doubtless the existence of photoreceptor molecules that may by their excitability incur liability for any light reaction. The increase of effect is increased by the steeper, expressly double-peaked curve, in which the photo-oxidative effect of the excellently excitating the xanten Rose bengale (conc. 1 : 50 000) is reflected. It is obvious that the photo-induced animal faster reaches the maximum, the value of which is much higher than that of the unstained one.

On the basis of the observed experimental facts we cannot help thinking of the photoinduced structural change of the cell *Tetrahymena* and, on the other hand, of the O_2-consumption that expresses in some degree the intensity of metabolism.

In the electronoptical photos of the unstained single organisms, light-treated for sixty minutes, there can essentially be recognized the structural relations of the not light-treated ones, the mitochondria being perhaps somewhat more swollen. There is practically a similar situation in case of the sensitized, not light-treated organisms as well. There is no particular change induced in the intracellular structures by the presence of dye. This fact may be interesting because the so-called dark reactions of the dyes of considerable effect belong to the questions of photobiology that have given rise to much controversy. There is remarkable lonely the deformation of nuclear envelope. The outer and inner membranes of the envelope have sporadically become loose, enclosing areas of considerable size (pathological perinuclear areas). In addition the density of cytoplasm is a little irregular. In case of the light-treated ones, however, we can observe definite changes.

First of all, the mentioned change of the nuclear envelope is attracting attention. In case of its dyed, light-treated organisms, taken from four to five days old cultures, we can recognize perfectly clearly the "star"-shaped chromatin (which is a little

dispersed) granules interconnected by filaments ("dark" nuclei by Nillson 1970). We cannot notice on them any peculiar change. The situation is the same at the cortical membrane structures, the pellicle of them consisting of three unit-membrane-like layers does not show any mark of photoinduced destructions, supposed by some investigators (Blum 1941, Giese 1953) as a phenomenon preparing the permeation of xanthen dyes. In the organized cortical membrane structures a desorganization may only be observed when the photoinduced movement entirely ceases to be, the body is swollen and begins to be rounded off.

We have investigated with increased attention the structure of cilia, beacuse the decrease in speed of the movement of *Paramecium* exposed to the UV-irradiation was explained by Saier and Giese (1966) by the lesion of cilia. As a matter of fact, there isn't shown any change that could be held responsible for the decrease in movement, either by their membrane, central and peripheral fibrils, or by the basal body-part infiltrated into the cytoplasm, by the parabasal body consisting of tubular and amorphous components and being recognizable at its proximal end or by the postciliary microtubules. These are giving, anyway, some supply for mediation of the dispersed, somewhat osmiophilic granules that can sporadically be observed in the longitudinal section of cilia. However, these are perhaps the arms on the one side of the peripheral filaments.

The most obvious sites of the photo-oxidative damages are the mitochondria. It was noticed by a number of investigators, that the ovoid or long-shaped, finger-biscuit-like, oval organelle can be found at nearly every ciliary base, pressing often en masse against the inner membrane of pellicle. It seems obvious that they have an immediate role, among others, in the functioning of cilia. It may be supposed that the sensitors infiltrate easily between the molecules of double membrane of the mitochondia, possibly even into the molecules themselves, forming a connection with the enzymes of the respiratory chain (Chalazonitis 1964). This may be concluded mainly from the photo-lesions observed in the tubular cristae and sporadically in the inner membrane. The tubuli were often desintegrated almost entirely, the electron dense matter was strongly reduced. The phenomenon may often be observed already after the first maximum of the speed of movement being formed, on other occasions, however, only after the animal being almost immobile, as the animals become incapable of being reactivated in response to light (Biczók and Pardutz 1970).

Besides the above changes, the modification of the state of cytoplasm granulated with uniform thickness, density is worth while mentioning. There appear smaller and larger knots, aggregates, meaning not only the damage of granules, ribosomes but, as emphasizes by several research workers, also a change in the conformation, and possibly in a structure, of the polypeptids giving a basic structure to cytoplasm (Erdmann 1955, Hyman and Howland 1940, Datta 1960, Montgomery et al. 1961).

The movement of cilia is a process of consuming energy (Kamiya 1959) at which

the lesion of the mitochondrium that is responsible for the oxidative phosphorylation cannot be at all indifferent (Beyer 1960) as the ATP production ceases to be continued or, at least, it decreases in a high degree. This is confirmed by the O_2-consumption that follows the light -activated by stain i.e., sensitized reactions of movement, that may be compared with the lesions of mitochondria, resp. the change in the speed of movement. Initially it is greater but later on it decreases. According to the measurements in every fifteen minute, the decrease in the sensitized organisms is much steeper, as well as also the descending branch of the curve of speed of the movement. We were also looking forward to the effect of ATP, resp. cysteine added to the substrate of defined ionic composition, to the formation of the reactions of movement, respiration, and electronmicroscopic structure.

Without Rosa bengale the effect of ATP and cysteine was rather moderate. The maximum of the speed of movement (within 10–12 min) appeared somewhat later, the descending branch of the curve lost in its rise. Also the O_2-consumption was activated a little more by light (about 15%), particularly in case of cysteine (above 40%). That is not particularly striking because, as known, the respiration of the normal cells is stimulated by cystein and glutathion, the amino acids containing SH-groups (Eichel and Roth 1953).

The effect was not spectacular even as cysteine or ATP was administered simultaneously with Rosa bengale to the animals, or after some hours, immediately before being investigated. The shape of curves of the speed of movement was rather close to that of those photosensitized. At the same time, there seemed to be very much surprising the O_2-consumption of the photosensitized cysteinic matter, being more than near four times higher than that of a similar matter without cysteine and sensitizer. How can be interpreted these results of the experiment?

The modest effect of ATP is comprehensible. It is known that if the energy store produced by respiration is filled up, the anorganic phosphate concentration is low, while at the low level ADP the respiration becomes slower. The speed of respiration is, however, controlled by ADP and the anorganic phosphates in the intact cells. A quick movement is elicited by the stress-effect of light particularly in the sensitized forms. The common action of ADP and anorganic phosphates, the energy production, i.e., the ATP synthesis are shown by the respiration being increased. As a result of the lesion of mitochondria, the connection between phosphorylation and respiration is broken off. It is not impossible that the moderate increase in the respiration is connected with that. The enormous O_2-consumption resulting from the joint effect of Rose bengale and cystein does not seem, as shown by the curve of the speed of movement, to be a controlled process, is, however, a different phenomenon. It can be explained, therefore, only with some difficulty. And as to the modest effect of ATP taken into the substrate, we have to think of that even the enzymes can be damaged which assure the liberation of the chemical energy of ATP appearing in the movement.

All these cannot be easily read off, of course, from the electronmicroscopic struc-

tures. A productive effect on the tubular cristae is exerted by ATP, a little by cystein, however, in a more striking way. This cannot be an indifferent phenomenon if we want to explain the minor increase in the O_2-consumption without dye in the presence of these substances.

There was a new programme in our investigation: the study of the effect of temperature, apart from that of light. It is known that the absorption of light by the photoreceptor molecules leads to a much quicker vibration or electronic excitation, needing much higher energy than that produced by heat. Nevertheless, we cannot leave out of consideration the heat induced quicker movements, that may lead to the disintegration of chemical bonds, to an easier, faster course of chemical reactions. Let us think that reaction taking place at 20°C passes off at 100°C 2^8, i.e., 256 times faster (van't Hoff's rule). It is understandable that the metabolism and throughout it the light sensitivity are influenced by temperature both quantitatively and qualitatively. For that we have several examples, among others the eggs of *Ascaris* and *Drosophila*, the ray sensitivity of which has risen after increasing the temperature of their vicinity (Holthusen 1921, Packard 1930). The most striking phenomenon is doubtless the increase in respiration reaching its maximum, according to our investigations on *Tetrahymena*, on average at 40°C. A peculiar consequence of that was seen at the movement and in the electronmicroscopic structure of animals photoactivated at a higher temperature.

We have observed that in case of rising temperature, the Ciliates sensitized with Rosa bengale, as a result of light, have reached the maximum of the speed of movement in shorter and shorter time; at 40°C in 4–5 min instead of the usual 10–12 min. The unstained *Tetrahymenae* that had tolerated almost unlimitedly the monochromatic light if the conditions of the environment were in other respect optimal, at 40°C after reaching a considerable speed perished in thirty minutes. The answer was given unequivocally by the electronmicroscopic pictures. The very characteritic sections made with the same procedure, simultaneously with earlier preparations were full of mitochondria being entirely or nearly entirely free of their cristae. And that the cell reacted to the more composed effect with almost all its parts, was shown by the fact that the parts had on large scale lost their ability to bind osmium, and the osmium granules of the membranes showing otherwise a strong resistance had demonstrated signs of desorganization.

Our results are referring to the quality of the participation of the cellular structures and mainly of the mitochondria and metabolism in the ciliary motion. The presence of sensitor touches the process of electron transport taking part in the respiratory chain of mitochondria through the change in oxygen requirement. The joint effect of the sensitor and cysteine, the high oxygen requirement that does not bring about any major change in the motion, have drawn our attention to an uncoupler-like phenomenon between oxidation and phosphorolysis. Its mechanism is, however, not clear for the time being.

Summary

We were looking for connection between the intensity of O_2-consumption, the speed of movement and the electron microscopic structure of the dye-sensitized, lighted *Tetrahymena pyriformis* influenced by ATP, cysteine and temperature. The results are referring to the quality of the participation of the cellular structure and mainly of the mitochondria and metabolism in the ciliary motion. The presence of sensitor touches the process of electron transport taking part in the respiratory chain of mitochondria through the change in O_2-requirement. The joint effect of the sensitor and cysteine, the high oxygen requirement that do not bring about any major change in the movement have drawn our attention to an uncoupler-like phenomenon between oxydation and phosphorylation.

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At this session has been presented also the paper of R. Rikmenspoel "Contractile mechanism in Flagella" published in Biophysical Journal 11, 446–463.

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