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CONTROL OF CELL SHAPE BY CALCIUM IN THE EUGLENOPHYCEAE

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SUMMARY

The euglenoid flagellates are able to change their shape rapidly in response to a variety of stimuli, or sometimes spontaneously. Two extremes of shape can be identified: the 'relaxed' form is cylindrical; the contracted form is a somewhat distorted disc. These 2 forms can be interconverted by treatments that alter the Ca^{2+} concentration of the entire cell. The level of Ca^{2+} is believed to be normally controlled by a system of calcium-accumulating membranes, identified in *Astasia longa* by the technique of calcium oxalate precipitation. The system forms a set of parallel tubes of endoplasmic reticulum, one of which lies immediately below each of the ridges of the pellicle. The individual ridges, each with its associated reticulum, microtubules and other elements are suggested to be independent motor units. Local activation of a small number of these units by Ca^{2+} is made possible by the arrangement of Ca^{2+}-sequestering reticulum, producing the characteristic squirming euglenoid movement. Uniform activation or suppression of all units produces the 2 extremes of shape.

The pellicle of *A. longa* with its associated microtubules has been purified and shown to contain a Ca^{2+}-binding site and ATPase activity.

INTRODUCTION

Unicellular algae of the class Euglenophyceae are well known for their characteristic changes in cell shape. These take the form of aperiodic contractions and re-extensions, referred to as euglenoid movement. The morphology of some of the more active species in this class shows a basic cylindrical symmetry, yet the cells are capable of deforming their surface into highly irregular asymmetric shapes. The structures that generate this motion have not yet been identified. Small groups of parallel microtubules lie just beneath the surface membrane and have been included in speculation about the elements responsible for the change of shape (Hofmann & Bouck, 1976; Leedale, 1964; Roth, 1959).

Several important aspects of the behaviour of eukaryotic cells seem to involve co-operation between microtubules and the surface membrane (Goldman, Pollard & Rosenbaum, 1976; Sommer, 1965). Euglenoid movement thus appears to be worth studying both as an example of cell motility and for what it may reveal about processes involving microtubules and membranes common to many other types of cell. In the course of investigations of the structural basis of this motion, I discovered several regimens that would induce all the cells to adopt the same shape. This led to the series of experiments reported here, which suggest that euglenoid movement is regulated by the intracellular calcium ion concentration.

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MATERIALS AND METHODS

Calcium ionophore A23187 was obtained from Calbiochem-Behring Corp., La Jolla, California. Spurr resin was supplied by E. M. Scope Laboratories Ltd, Ashford, Kent. Percoll® is a product of Pharmacia, Uppsala, Sweden. Aquasol-2® is produced by New England Nuclear, Boston, Massachusetts.

Organisms and culture conditions

Strain numbers refer to the collection of the Culture Centre of Algae and Protozoa (CCAP), 36 Storeys Way, Cambridge. *Euglena gracilis* (CCAP 1224/5g) and *Astasia longa* (CCAP 1204/17d) were grown in axenic culture, the former in the *E. gracilis* medium of the CCAP, the latter in this medium plus 1 % protease peptone, or in the modified Cramer–Myers medium described below. Cells were harvested during log-phase growth. *Euglena spirogyra* (CCAP 1224/13a) and *Distigma proteus* (CCAP 1216/3a) were grown on the soil and water 'medium E' described in the CCAP catalogue. Small samples of *Eutreptia viridis* (CCAP 1226/1c), *Peranema tricophorum* (CCAP 1260/2), *Phacus pleuronectes* (CCAP 1261/3b), *Trachelomonas hispida* (CCAP 1283/9) and *Colacium vesiculosum* (CCAP 1211/1) were obtained from the CCAP and used immediately without further subculture.

A modified Cramer–Myers medium (Padilla & James, 1960), with acetate substituted for 90 % of the sulphate, was used to grow *Astasia longa* prior to ^{35}S labelling. After 12 days growth in the non-radioactive medium, the cells were spun down and resuspended at about 1×10^4 cells/ml in fresh sterile medium containing 50 μCi/ml ^{35}S as $^{35}SO_4^{2-}$. After a further 75 h the cells were harvested. Under these conditions the doubling time was about 16 h.

Microscopy

Light micrographs were recorded on 35 mm black and white film, 20 ASA, using an electronic flash and a Zeiss standard photomicroscope with Nomarski differential interference contrast or dark-field optics.

Electron micrographs were taken on a Philips EM400 microscope at 80 kV (stained thin sections) or 60 kV (unstained sections). X-ray microprobe analysis was attempted on a Philips 400 EDS system (kindly made available by Dr E. Munns, A.R.C. Animal Physiology Unit, Babraham, Cambridge) and on a JEOL 100 CX-TEMSCAN (courtesy of JEOL (U.K.) Ltd, JEOL House, Grove Park, Colindale, London, N.W.9).

Samples were fixed by additions of glutaraldehyde in buffer A (see below) to a final concentration of 2·5 %. After 1 h at room temperature the specimen was washed into 0·1 M-collidine, pH 7·0 and treated with 0·4 % OsO_4 for 30 min on ice, dehydrated with anhydrous ethanol, exchanged into propylene oxide and embedded in Spurr resin.

Sections were cut with a diamond knife, stained with 5 % uranyl acetate at 60 °C for 20 min followed by lead citrate at room temperature for 5 min. In the experiments with lead phosphate or calcium oxalate precipitates, thick sections (purple-gold) were used without staining.

Localization of calcium storage sites

Astasia longa was harvested from culture medium and suspended in 10 mM-$CaCl_2$ for 30 min. The cells were washed 4 times with 50 vol. of distilled water and then put in 10 mM-potassium oxalate, pH 6·8 for 15 min. After washing to remove oxalate, the cells were returned to the calcium chloride solution. This cycle was repeated 3 times, after which the cells appeared normal in the light microscope. They were immediately fixed, in the presence of 10 mM-oxalate, and prepared for sectioning as described. As a control, cells were exposed to 10 mM-$CaCl_2$ alone and also to distilled water alone.

Localization of ATPase activity

The lead citrate reagent for ATPase localizations was prepared immediately before use as follows. To 2·0 ml 0·1 M-potassium citrate was added 0·1 M-lead nitrate dropwise with stirring,

while holding the pH at 8·0 by addition of KOH as necessary. When the solution was just saturated with lead, the pH was lowered to 7·0 with HNO_3. A white precipitate formed (as some of the Pb^{2+} is displaced from citrate by H^+) and was removed by Millipore filtration followed by centrifugation at 44 000 *g* for 15 min. To the supernatant was added 0·1 ml 100 mM-potassium citrate, pH 7·0, to provide a small amount of free chelating agent as protection against non-specific precipitation of lead. Conditions that cause lead to precipitate from this solution are a drop in pH or addition of high concentration of divalent cation.

To 1 ml of pellicle suspension, 25 μl of the lead citrate reagent was added and stirred for 2 min at room temperature. ATP was added (25 μl of 80 mM, pH 7·0) and after 10 min, glutaraldehyde (pH 7·0) to a final concentration of 5%. The suspension was centrifuged (18 000 *g* for 5 min), resuspended in 1 ml buffer A and recentrifuged. The pellet from this second centrifugation was fixed for 2 h with 5% glutaraldehyde at 4 °C, treated with 1% OsO_4 for 1 h and then broken into a few pieces for embedding as usual.

The sartorius muscle of *Rana pipiens* was used as a test specimen for this reagent. A muscle mounted at rest-length on a Perspex strip was made permeable by the 'fast glycerination' procedure of Rome (1973). The muscle was then washed in buffer plus 1 mM-$CaCl_2$ for 24 h and treated with lead citrate and ATP as above. After 10 min, the muscle was washed with stirring in 2·5% of glutaraldehyde in buffer for 1 h. A few fibres from the surface of the muscle were dissected free and carried through the normal fixation and embedding procedure.

Preparation of subcellular fractions

Cells were deflagellated as described by Hofmann & Bouck (1976), washed extensively to remove free Ca^{2+}, then resuspended in 50 mM-KCl, 5 mM-$MgCl_2$, 50 mM-PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)), pH 7·2, 0·1 mM-dithiothreitol, 0·1 mM-phenylmethylsulphonyl fluoride, 0·1 mM-tosylarginine methyl ester, 1 μg/ml pepstatin (henceforth buffer A). Mild sonication or fracture in a Kontes cell disruptor (Kontes Inc., Vineland, New Jersey) was used to break the cells, which were then centrifuged at 5000 *g* for 10 min.

Pellicle was separated from this pellet by fractionation on a Percoll density gradient (70% Percoll in buffer A) at 40 000 *g* for 20 min. Further purification was achieved by removing the pellicle band from this gradient, washing it several times by low-speed sedimentation (200 *g*) in buffer A, then repeating the density gradient fractionation on 70% Percoll. The purified material was washed to remove Percoll by washing 10 times with 20 vol. buffer A. In the purification of material used for the experiments with ouabain, Na^+ was substituted for K^+ in making up all components of buffer A.

The microsomal fraction of *E. gracilis* was prepared from cells fractured in buffer A plus 250 mM-sucrose in the cell disruptor. The presence of sucrose prevented chloroplast breakage. The broken cell suspension was centrifuged for 15 min at 15 000 *g*. The supernatant from this spin was pelleted at 100 000 *g* for 1 h. The pellet was resuspended in a small volume of buffer A and centrifuged briefly at 18000 *g* to remove aggregated material. It was used immediately, as the Ca^{2+} transporting activity was lost in about 12 h.

Ca^{2+} binding assays

A 75-μl sample was mixed with 75 μl buffer A containing 20 μM-$^{45}Ca^{2+}$ and incubated for various times, as indicated in Results. Unbound Ca^{2+} was removed by filtration on Millipore filters, 1·2 μm pore size, followed by 2 washes with 0·5 ml Ca^{2+}-free buffer A. The filters were dried, dissolved in 0·5 ml methyl cellosolve, and counted in 5 ml Aquasol-2® using the standard ^{14}C energy discriminator settings. There was a small reproducible background due to non-specifically retained ^{45}Ca, about 500 c.p.m. In some experiments, the samples with bound Ca^{2+} were removed from solutions by pelleting at 100 000 *g* for 10 min, using in this case [^{3}H]glucose as a volume marker. The pellets and tubes were rinsed twice before resuspending the pellet in Aquasol and counting as above. This method gave a lower background than filtration, but did not allow precise control of the incubation time. Allowing for these factors, the 2 methods gave essentially identical results.

ATPase assay

The procedure of Seals, McDonald, Bruns & Jarett (1978) was used to measure hydrolysis of [γ-^{32}P]ATP. A 250-μl pellicle suspension was mixed with various components plus buffer A to a final volume of 310 μl. In the assays with ouabain, Na^+ replaced all but 1 mM of the K^+ in the medium; with EDTA, Mg^{2+} was omitted. When metavanadate was used, it was incubated with the sample for 1 min before adding ATP. In all cases, the reaction was initiated by adding 7 μl 70 mM-[γ-^{32}P]MgATP. After 1, 2, 5, 10 and 30 min, 50-μl aliquots were removed, mixed with 10 μl 10% sodium dodecyl sulphate to terminate the reaction, and assayed for $^{32}P_i$. The reaction had a linear time course until about 20% of the ATP had been hydrolysed. Rates were calculated from the slope of the linear part of a graph of total extractable counts *versus* time. The control hydrolysed 11·1 nmol ATP/mg protein per min.

RESULTS

Experiments with whole cells

In the most widely studied species of the Euglenophyceae, *E. gracilis*, the undisturbed freely swimming cell resembles a somewhat flattened cylinder. Following a variety of mechanical or chemical stimulants, or sometimes apparently spontaneously, the cell alters its shape in a way which varies from simple bending in one plane to a complicated simultaneous shortening, twisting and bending. Often the cells continue a very fluid squirming motion for many minutes without pause. The most extreme change produces a cell resembling a disk folded or rolled up from opposite sides until the edges meet in the middle. Fig. 1 shows cells responding in this way to a sudden increase in illumination. A search was undertaken for methods of producing a homogeneous population of cells permanently in either their most elongated or fully contracted form. A consistently effective regimen found was treatment with caffeine. Within a few minutes of exposure to 10 mM-caffeine practically all cells became highly active, with many irregular contractions, finally taking up the folded disk shape (Fig. 2). This effect is reversed by washing out the caffeine and can be repeated through several cycles of addition and removal. Fig. 2 shows cells in the resting state (A) and after caffeine treatment (B). In these 2 situations the cells retain their approximate cylindrical symmetry. Fig. 2C–F illustrates transient forms observed during normal euglenoid movement. A characteristic feature of this motion is local variation and reversal of membrane curvature, giving an asymmetrical overall shape.

In the cells of vertebrates, caffeine is known to have at least 2 independent actions. It has the effect of raising cyclic nucleotide levels by acting as a competitive inhibitor of cyclic nucleotide phosphodiesterase (Butcher & Sutherland, 1962). It also has the ability to cause contraction of muscle cells, which results from a release of calcium stored in the sarcoplasmic reticulum (Weber & Herz, 1968). In *Euglena* the caffeine effect appears to be mediated by calcium rather than cyclic nucleotides (Table 1). The cells do not respond to addition of cyclic nucleotides, nor do these compounds influence their response to caffeine. The caffeine response is, however, mimicked, in the presence of external calcium, by the calcium ionophore A23187. It is also mimicked by the detergent Nonidet P-40, which presumably makes the cell membrane directly permeable to calcium. The Ca^{2+} effect is reversible: cells that elongate after treatment

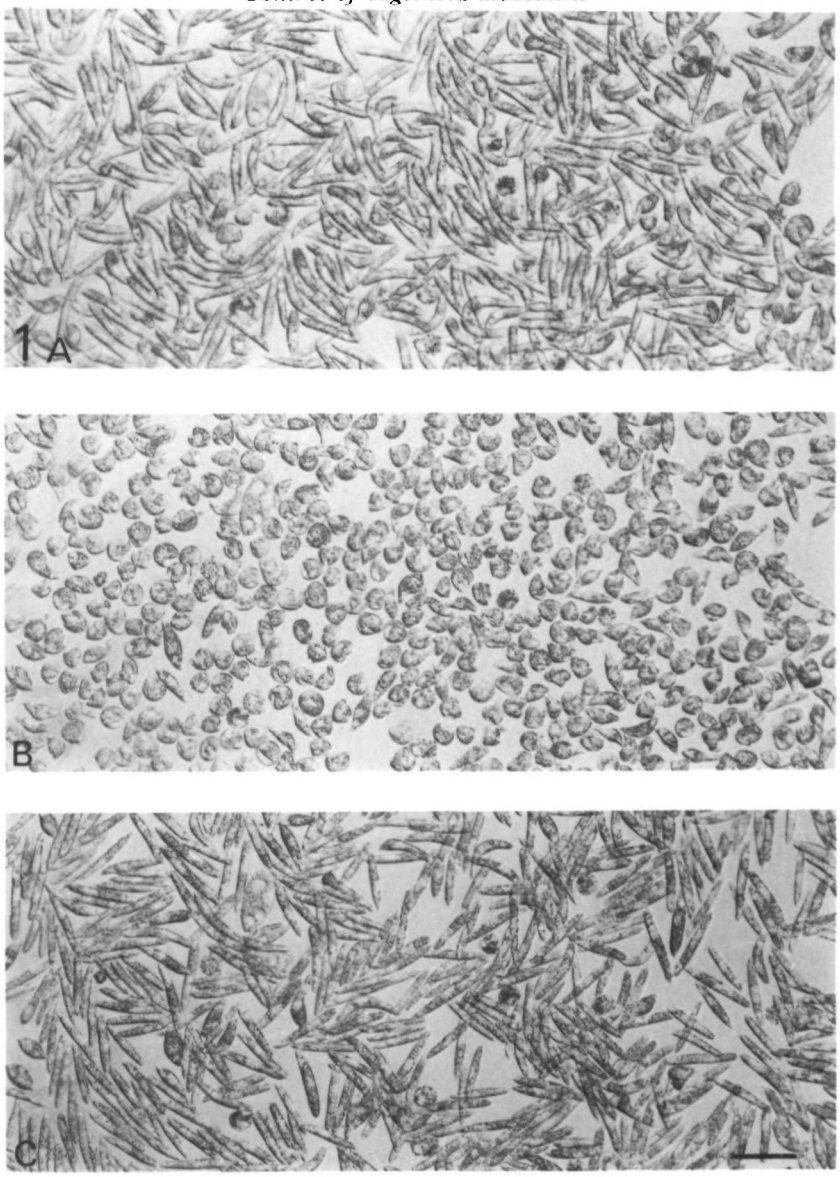


Fig. 1. Shape changes in *E. gracilis* in response to changing light intensity. Cells were exposed to a sudden increase in illumination by turning up the voltage to the microscope lamp at time zero, and then turning it down again after 5 s. Photographs were taken: A, approximately 0·5 s after the increase in illumination, before the cells responded; B, 3 s after increase; C, 10 s after decreasing illumination, i.e. 15 s after A. Nomarski interference contrast optics. Bar, 50 μm. × 160.

with A23187 plus EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid) contract when the Ca^{2+} level is raised and *vice versa*. The effect is specific for Ca^{2+}; Mg^{2+} at the same or higher concentrations cannot be substituted for Ca^{2+}.

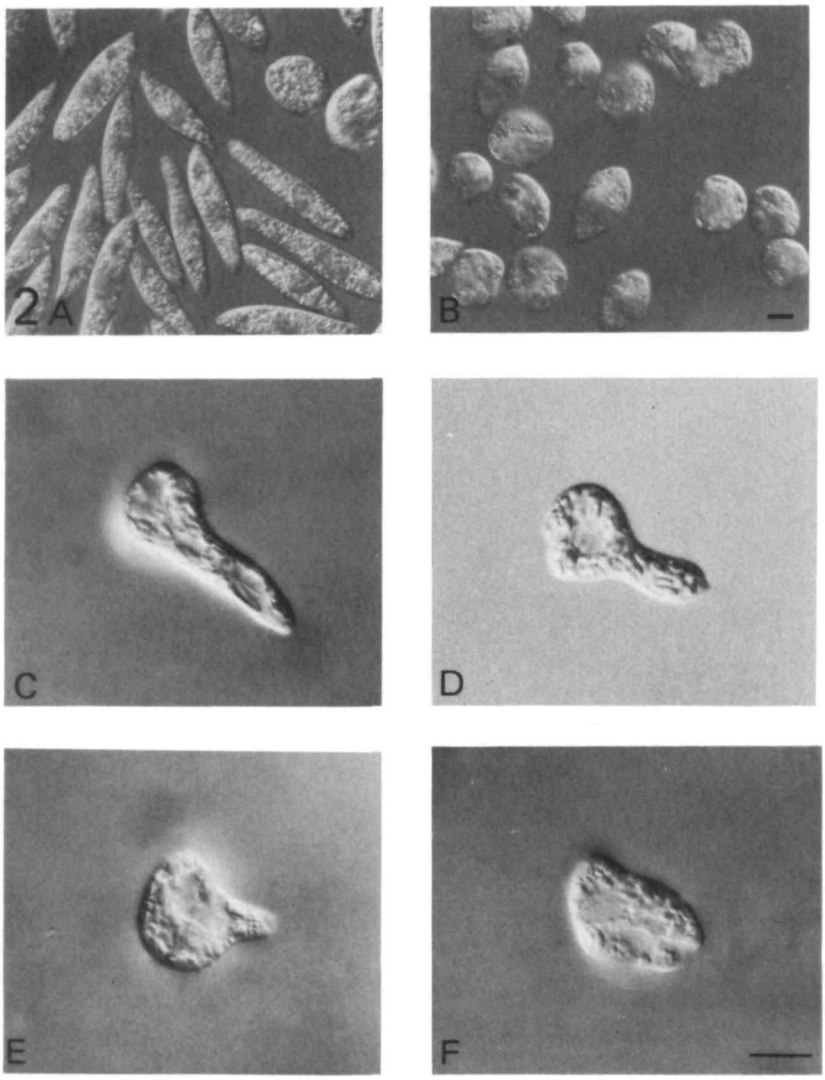


Fig. 2. Euglenoid movement. Cells are shown in the resting, elongated state (A), completely contracted after 5 min exposure to caffeine (B), and during normal asymmetric contractions (C–F). Bar, 10 μm; A, B, ×300; C–F, ×810.

Two other features of interest are that, with caffeine external calcium is not required, and the response is energy-dependent. Cells poisoned with cyanide or dinitrophenol do not respond to any of the stimuli mentioned above.

The role of Ca^{2+} in regulating cell shape is not limited to this species. Within the genus *Euglena*, identical responses to Ca^{2+} were produced in *E. spirogyra*. Similar responses were also found in members of several other genera, including both sapro-

phytic and photosynthetic forms (*Astasia longa*, *Distigma proteus*, *Eutreptia viridis*, *Peranema tricophorum*, *Colacium vesiculosum*). In *Phacus pleuronectes* (a species believed to be non-motile; Mignot, 1965), no response was seen, and in *Trachelomonas hispida* (observing motion within the lorica) the results were equivocal.

Table 1. *Effect of various reagents on the shape of* E. gracilis

Treatment	Distribution of shapes (%)		
	Elongated	Intermediate	Fully contracted
No addition	40–80	10–30	10–30
1·5 mM-cAMP	30–60	10–30	10–30
1·5 mM-dibutyrl cAMP	30–60	10–30	10–30
1·5 mM-dibutyrl cGMP	30–60	10–30	10–30
10 mM-caffeine	6	4	90
10 mM-caffeine + 10 mM-EGTA	4	2	94
20 μg/ml A23187, 0·1% NP40, 10 mM-$CaCl_2$	4	11	85
20 μg/ml A23187, 0·1% NP40, 10 mM-$MgCl_2$ + 10 mM-EGTA	85	10	5
20 μg/ml A23187, 0·1% NP40, 10 mM-EGTA	84	9	7

Cells were washed twice then resuspended in H_2O. Reagents were added to the indicated concentrations and after 20 min the cell suspension was examined at × 160. Roughly 100 cells were examined and classified as follows: elongated, similar to the predominant form in Fig. 1A; fully contracted, the predominant form in Fig. 1B; intermediate, cells partially contracted or bent but not yet disk-shaped. Results are sometimes given as a range to indicate that under these conditions the cells are actively changing shape and the observed distribution is likely to fluctuate within these boundaries.

A rough estimate was made of the concentration of Ca^{2+} required for activation in *E. spirogyra*. After treatments which make the cell permeable to Ca^{2+}, the fraction of cells that contracted increased with the concentration of Ca^{2+} in the medium (Fig. 3). By buffering the free Ca^{2+} with Ca-EGTA one finds that most of the transition occurs between 10^{-7} and 10^{-5} M-Ca^{2+} (Figs. 3, 4). In Fig. 4 the fraction of disk-shaped cells is plotted against free [Ca^{2+}]. The solid line was calculated for a calcium dissociation constant of $2{\cdot}2 \times 10^{-6}$ M.

The simplest interpretation of these experiments is that Ca^{2+} activates the apparatus that produces euglenoid movement. It is reasonable to suppose that the normal regulation of movement also involves changes in the concentration of Ca^{2+} somewhere in the cell. The 'resting', elongated, cell thus has fixed its [Ca^{2+}] at a low level, probably less than 10^{-7} M. The response to caffeine suggests that each cell maintains an internal store of Ca^{2+}, in sufficient quantity to activate its motile apparatus. The next experiments were carried out in order to locate within the cell the system that transports and accumulates calcium. Similar systems have been identified in other motile cells by using oxalate to precipitate Ca^{2+} (Hasselbach, 1964; Constantin, Franzini-Armstrong & Podolsky, 1965; Etienne, 1970). The calcium salt of oxalic acid has a solubility product of $1{\cdot}8 \times 10^{-9}$, much lower than the solubilities of its

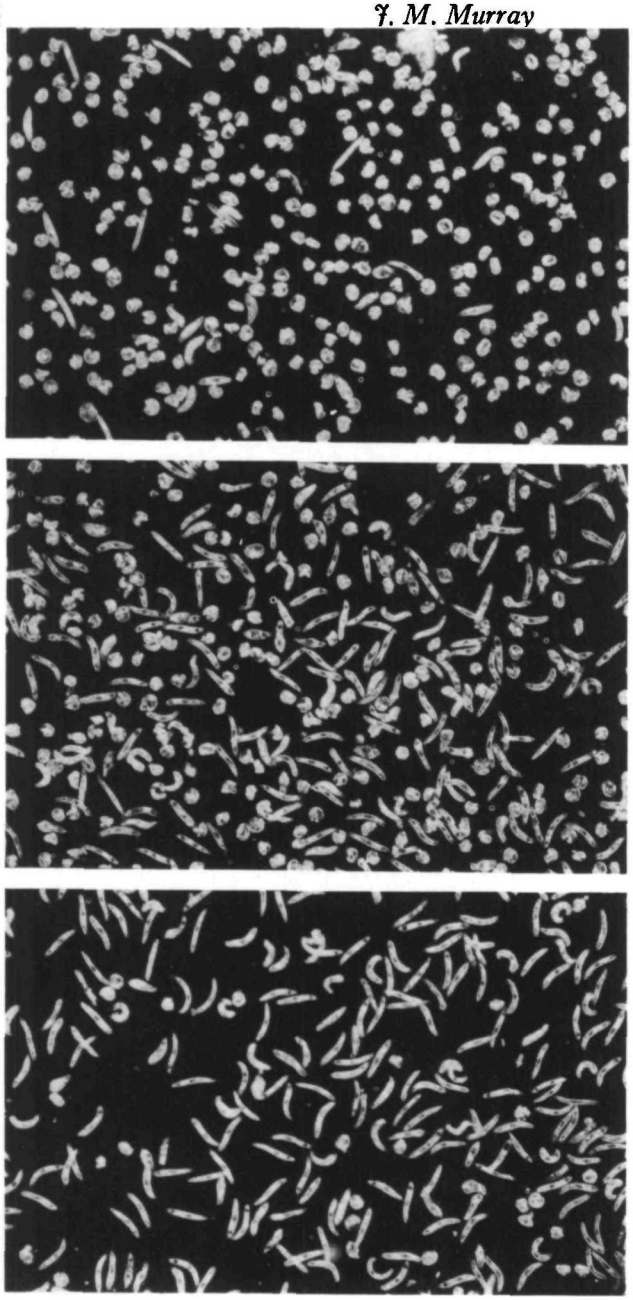


Fig. 3. Shape change in *E. spirogyra* induced by Ca^{2+}. Washed cells were suspended in a mixture of 0·0013% Nonidet P40, 33 μg/ml A23187, 4 mM-PIPES, and 4 mM (EGTA + Ca-EGTA). The ratio of EGTA to Ca-EGTA was adjusted to give the free calcium concentrations recorded on the right. Photographs were taken after 20 min. Bar, 150 μm. ×60, dark-field optics.

salts with the other common intracellular cations (magnesium oxalate, $K_{sp} = 8{\cdot}6 \times 10^{-5}$; sodium oxalate, $K_{sp} = 0{\cdot}5$; potassium oxalate, $K_{sp} = 1{\cdot}4$). In accordance with this, the precipitates formed after oxalate treatment of cells have been shown to be calcium by X-ray microanalysis (Podolsky, Hall & Hatchett, 1970; McGraw, Somlyo & Blaustein, 1980). When *A. longa* is treated with oxalate, small electron-dense precipitates are formed intracellularly. A few granules can be seen in the mitochondria, but the majority are found in the channels of endoplasmic reticulum, which lie just beneath the surface membrane (Fig. 5A–C). The arrangement of

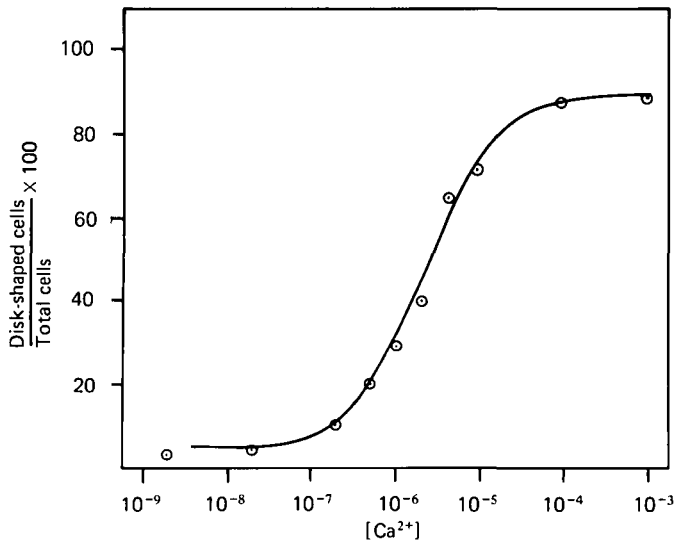
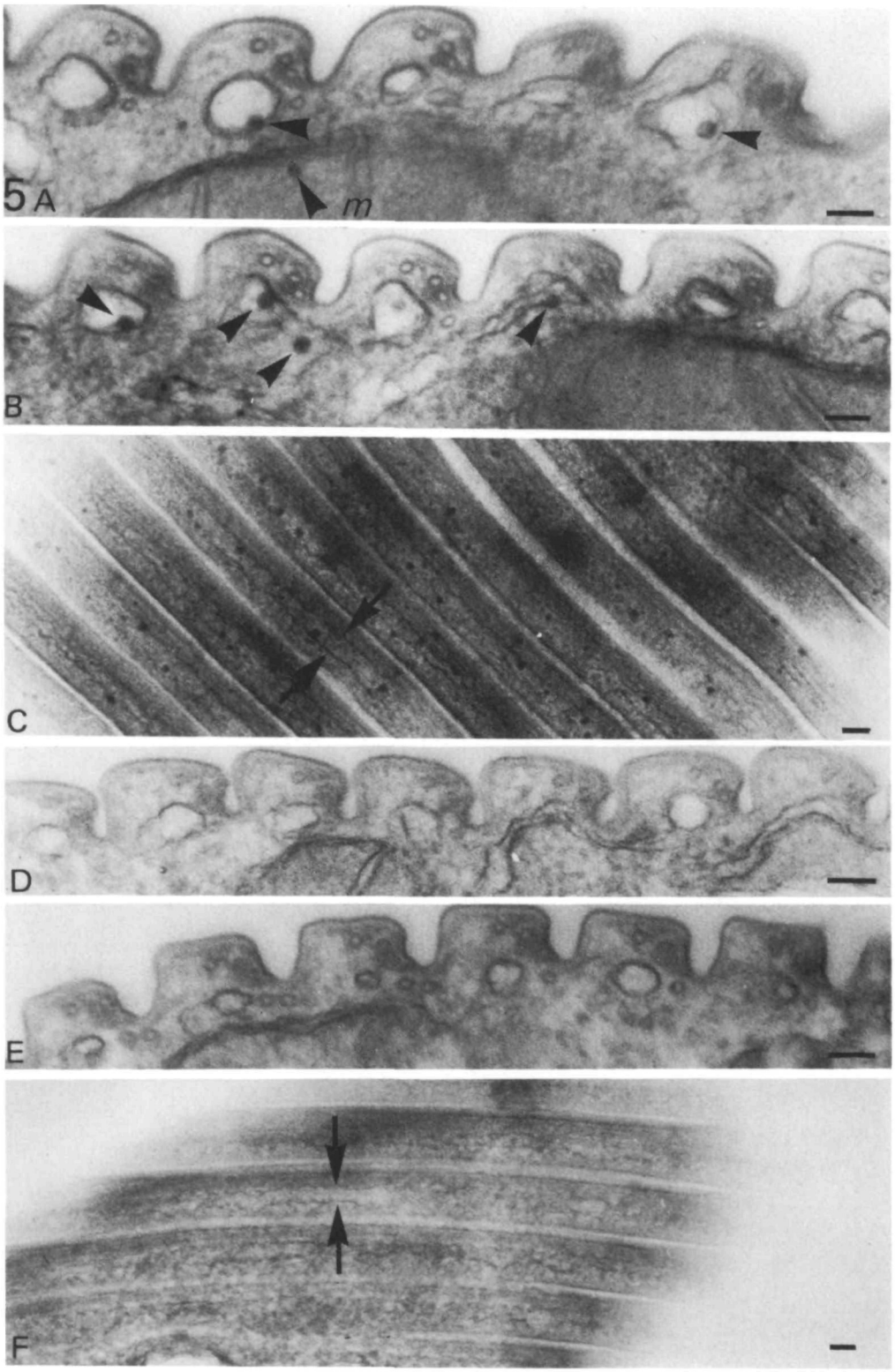


Fig. 4. Distribution of cell shapes as a function of Ca^{2+} concentration. Cells treated as for Fig. 3 were photographed, and the fraction that was disk-shaped determined. A single point represents an average of 2 determinations, each of about 500 cells. The line is calculated for a dissociation constant of $2{\cdot}2 \times 10^{-6}$ M, with a range of 5 to 90% disk-shaped.

this reticulum is shown schematically in Fig. 6. It comprises a set of interconnected tubes, one of which runs beneath each ridge of the pleated surface of the cell. There are occasionally anastomoses between neighbouring parallel tubes, and branches that lead to the reticulum deeper in the interior of the cell (Ringo, 1963).

In Fig. 5C, one can see that the granules formed by oxalate treatment lie neatly in rows within the channel of endoplasmic reticulum below each pellicular ridge. In the control cells, not treated with oxalate, no granules are found (Fig. 5D–F).

The precipitates formed in *A. longa* by oxalate treatment are smaller than those analysed in other cells. Assuming the granules are pure calcium oxalate, spherical and 200 Å in diameter, they are expected each to contain about 2×10^{-17} g of calcium. Several attempts were made to analyse granules by electron microprobe, but as might be expected this is below the detection limit of commercially available X-ray microprobe analysis instruments. This is a very small amount of Ca^{2+}, but if the granules, which are spread on average about 2000 Å apart, represent previously


5 A
m
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dissolved Ca^{2+} then the initial concentration in the reticulum is calculated to have been over 10 mM.

Experiments with subcellular fractions

The previous experiments with whole cells suggest the presence of a motile system whose activity is regulated by cellular control of calcium concentrations, and a well developed internal Ca^{2+} transport system. An attempt has been made to isolate the calcium-accumulating membrane. A microsomal fraction of *E. gracilis* was prepared and assayed for its ability to transport Ca^{2+}. The preparation exhibits ATP-stimulated Ca^{2+} uptake against a concentration gradient, and releases its bound Ca^{2+} on exposure to caffeine (Table 2). A homogeneous membrane fraction would not be expected from the simple preparative procedure used, and Fig. 7 confirms this expectation. The microsomal preparation contains non-membranous elements as well as 2 or more distinguishable types of membrane. EGTA almost completely inhibits uptake, ruling out the possibility of some non-specific physical entrappment within the particulate material. The very slow association of Ca^{2+} with the particulate fraction indicates that this is not simple stoichiometric binding to an ordinary binding site, on the outside of the vesicles for instance. Ca^{2+}-specific binding sites of the ordinary sort typically come to equilibrium with ionic calcium in milliseconds (Eigen & Hammes, 1963). The radioactivity associated with the vesicles is not removed by washing with Ca^{2+}-free buffer (see Materials and methods for a complete description of the assay), a finding which is also incompatible with a simple Ca^{2+}-binding site. One might argue that tightly bound Ca^{2+} would not be removed by these washes, but the argument can be rejected for 2 reasons. First, the uptake system has a perfectly ordinary affinity for Ca^{2+}, since uptake is abolished by EGTA but proceeds well at 2×10^{-5} M free calcium. Second, this argument confuses 'tightly bound' with non-exchangeable. The rate constant for dissociation of Ca^{2+} from even very high-affinity protein binding sites is commonly much faster than 1 s^{-1} (Eigen & Hammes, 1963). Thus complete removal of radioactivity bound to any ordinary stoichiometric binding site would be expected during the course of a washing procedure of several seconds.

Could the uptake into the vesicles be passive transport? The volume enclosed within the vesicles is a small fraction of the total assay mixture, certainly less than 1 %. In the course of the 5-min assay, more than 15 % of the Ca^{2+} was sequestered in

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Fig. 5. Sections of oxalate-treated *A. longa* showing calcium oxalate deposits in endoplasmic reticulum and mitochondria of untreated cells lacking deposits. The somewhat fuzzy appearance of these micrographs is a consequence of using unstained, rather thick sections. (A, B) Transverse sections of oxalate-treated cells with granules indicated by arrowheads. *m*, deposit within a mitochondrion. (C) Glancing section of an oxalate-treated cell, showing granules mainly confined to the channel of the subpellicular endoplasmic reticulum. The walls of the reticulum are seen in projection as 2 narrow wavy black lines, indicated at one point by arrows. (D, E) Transverse section of untreated control cells; no deposits are present. (F) Control cell, glancing section; no deposits present. The arrows point to the wall of the endoplasmic reticulum. A, B, D, E ×80000; bar, 0·1 μm. C, F ×37000; bar, 0·1 μm.

the vesicles. This cannot be simple passive transport, since it creates a concentration gradient. It demands energy expenditure, hence the stimulation by ATP. The reversal of uptake by caffeine also argues strongly against passive transport. The uptake of Ca^{2+} seen without added ATP is simply explained by the presence of either residual cellular ATP not washed out during the preparative procedure or some other

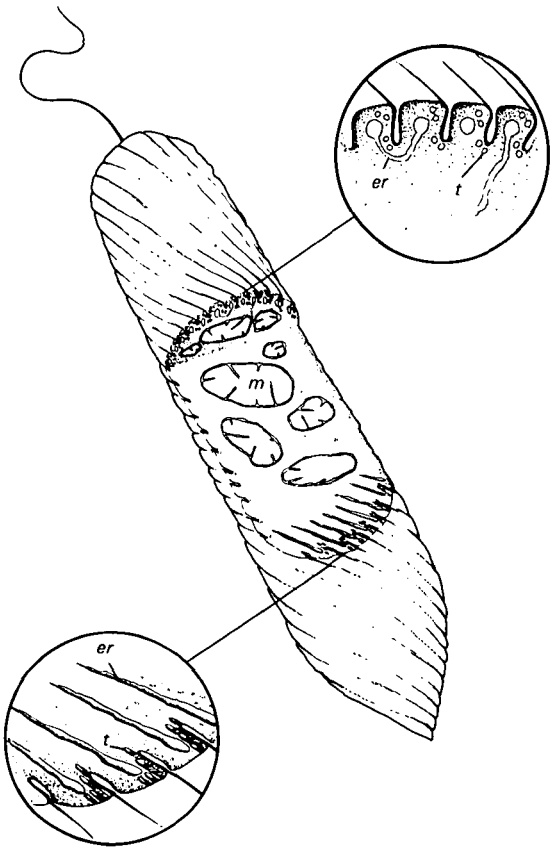


Fig. 6. A diagram of the pellicle and subpellicular components of *A. longa*, illustrating the planes of Fig. 5A–B (upper circle) and Fig. 5C (lower circle). *m*, mitochondrion; *er*, endoplasmic reticulum; *t*, microtubules.

substrate that can be converted into utilizable energy by the fragments of mitochondria in the preparation. The uptake is partially blocked by oligomycin, which suggests that part of the uptake may be due to contaminating mitochondrial fragments. A significant proportion must be due to other types of transport system however, since mitochondria are insensitive to caffeine (Weber, 1968). Further purification steps have so far led to loss of activity, so that it is not yet possible to identify certainly the intracellular organelle that gives rise to the microsomal transport system. It should be noted, however, that the cells distinctive pellicle is not present in the microsomal fraction.

The architecture of the pellicle complex of *Euglena* has been described by several

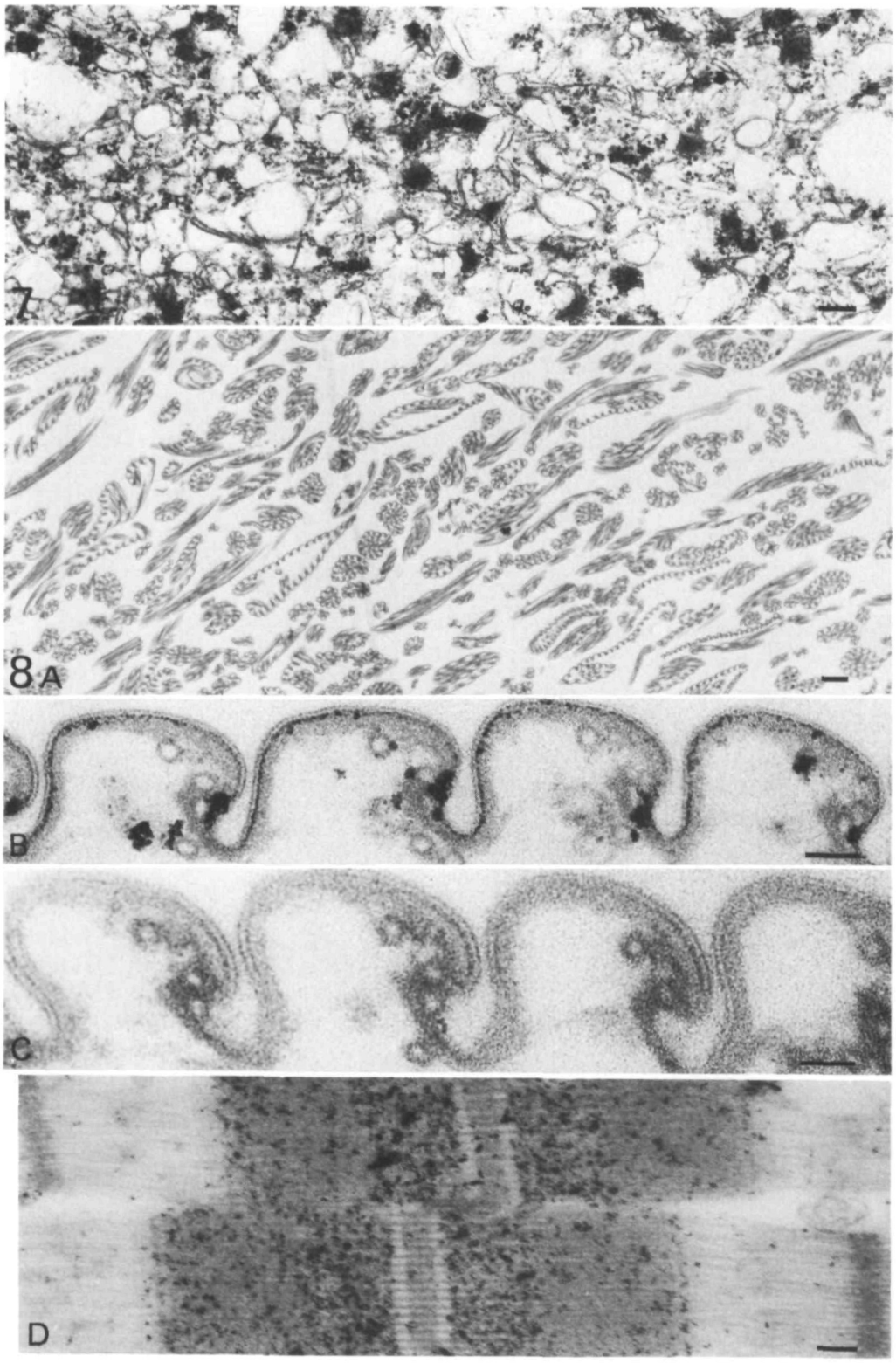
authors (Kirk & Juniper, 1964; Leedale, 1964; Sommer, 1965; Mignot, 1965; Lefort-Tran, Bre, Ranck & Pouphile, 1980) and its composition determined (Hofmann & Bouck, 1976). It is likely that one of the components of this complex is the apparatus responsible for euglenoid movement, but it has not yet been identified. One obvious candidate is the set of subpellicular microtubules which can be seen in Fig. 5 and which, with some variation in number, are a prominent feature of most if not all Euglenophyceae (Mignot, 1965). An alternative, less obvious in the species pictured here but certainly present in other species, is a group of filamentous structures that are also parallel to the plane of the membrane but oriented at an angle to the direction of the microtubules (Miller & Miller, 1978; Lefort-Tran *et al.* 1980).

Table 2. *Ca^{2+} binding by particulate subcellular fractions of* Euglena

Sample	Additions	^{45}Ca bound (c.p.m.)
(a) Microsomal fraction		
1 min incubation time	None	3314
5 min incubation time	None	13930
	Omit ATP	6612
	10 mM-EGTA	964
	50 μg/ml oligomycin	4882
	10 mM-caffeine, added after 4 min incubation	4610
(b) Purified pellicle	None	7409
	2·7 mM-MgATP	7287
	3 mM-MgATP + 7 mM-caffeine	6979

Binding was measured as described in Materials and methods. Assay conditions: (a) 0·1 M KCl, 5 mM-$MgCl_2$, 10 mM-imidazole, pH 7·0, 4 mM-MgATP, 23 °C; total added calcium $2{\cdot}1 \times 10^{-5}$ M, corresponding to $8{\cdot}5 \times 10^4$ c.p.m.; 0·06 mg protein per assay; (b) 50 mM-PIPES, 5 mM-$MgCl_2$, 10 mM-[^{3}H]glucose, pH 7·2, 20 °C; total added calcium 2×10^{-6} M, corresponding to $1{\cdot}5 \times 10^4$ c.p.m.; 0·3 mg protein per assay.

As a complement to structural studies on the pellicle and in the hope of learning something about the motile apparatus of these cells, a characterization of the biochemical and enzymic properties of isolated pellicle was undertaken, with 2 interesting results. First, the purified pellicle complex contains a tightly associated ATPase. The ATPase activity is Mg^{2+}-dependent, but only slightly Ca^{2+}-sensitive in the presence of 5 mM-magnesium. Dinitrophenol gives a slight activation, less than 20%, which may indicate a small contamination of the preparation with mitochondrial ATPase. Neither this nor other (e.g. glycolytic) contaminants contribute significantly to the observed ATP hydrolysis, however, as shown by the lack of inhibition by oligomycin (9 μg/ml) and azide (10 mM). Nor can the activity be explained as a membrane Na^+-K^+-ATPase, judging from the lack of response to 10^{-5} M-ouabain and low sensitivity to vanadate (5% inhibition by 2 μM-sodium metavanadate). The ATP hydrolysis was completely inhibited by *p*-chloromercuribenzoate, a sulphhydryl reagent. During ATP hydrolysis pellicle observed in the light microscope undergoes no discernible change of shape.


7
8A
B
C
D

The attribution of this ATPase activity to the pellicle itself and not to some other contaminant of the preparation is supported by 2 other pieces of evidence. First, the pellicle preparation contains very little except pellicle. Fig. 8A shows a low-magnification view of a section of the pellet obtained after centrifuging the pellicle preparation at 100000 *g* for an hour.

Secondly, the site of ATP hydrolysis has been visualized by the precipitation of lead phosphate, using a modification of Gomori's procedure (Gomori, 1952). In Fig. 8B the precipitate is seen along the membrane and around a microtubule associated with the pellicle. The control, Fig. 8C, in which no ATP was added, shows no precipitate. A further control was carried out to guard against the possibility that the pellicle might adsorb granules of lead phosphate precipitated elsewhere in solution. A small amount of inorganic phosphate was added to the control containing pellicle and lead solution but no ATP. It was found that very little of the precipitated lead phosphate stuck to the pellicle. The few granules that were observed seemed to have become lodged in the narrow groove between adjacent pellicular ridges. This, of course, is on the extracellular surface of the pellicle, unlike the precipitate seen in Fig. 8B. The specificity of this precipitation reaction is demonstrated by using glycerinated vertebrate skeletal muscle as a test object (Fig. 8D). The lead precipitate is seen over the regions of the thick filament that bear cross-bridges and in remnants of the sarcoplasmic reticulum. Since this corresponds with the known location of ATPase sites in muscle and very little precipitate occurs elsewhere, there can have been no significant diffusion of phosphate away from the hydrolysis sites before precipitation, nor any significant migration of the precipitated material during fixation, embedding and sectioning.

The second interesting property of purified pellicle is its possession of a specific high-affinity Ca^{2+}-binding site. The binding of Ca^{2+} at a medium concentration of a few micromoles occurs in the presence of 5 mM-Mg^{2+}. It is not ATP-dependent and is unaffected by caffeine, so it is unlikely to be due to uptake by an ion-transport system (Table 2). The amount of Ca^{2+} bound did not increase with longer incubation times. Ca^{2+} binding has very little effect on the pellicular ATPase.

It is not yet possible to specify which elements of the *Euglena* pellicle complex enable it to change its shape in such dramatic fashion. However, at least 1 possible candidate for this role can be eliminated. Purified pellicle run on sodium dodecyl

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Fig. 7. Microsomal fraction of *E. gracilis*. The material used for the Ca^{2+} transport assay described in the text was sedimented at 100000 *g*, fixed, and embedded as a pellet. Bar, 0·2 μm; × 32000.

Fig. 8. (A) Low magnification view of a section of the pellet formed by centrifuging isolated pellicle. Bar, 1 μm; × 3900. (B) High magnification view of pellicle treated for ATPase localization. The precipitate of lead phosphate is seen just under the cell membrane and in the region of the microtubules. Unstained section. Bar, 0·05 μm; × 144000. (C) Control experiment in which ATP was omitted. Unstained section. Bar, 0·05 μm; × 160000. (D) Test specimen for the ATPase localization procedure. Frog sartorius muscle. Precipitate is found over the region of the thick filament-bearing cross-bridges and in remnants of the sarcoplasmic reticulum. Unstained section. Bar, 0·1 μm; × 52000.

sulphate/polyacrylamide gels shows no trace of a peptide migrating with the molecular weight of actin. Cytoplasm also has very low (undetected on gels) amounts of actin and no cytoplasmic filaments resembling actin are seen in sectioned cells. An experiment was performed to determine how much, if any, actin is present in one species. *A. longa* can be grown on a chemically defined medium. The proteins of the cell can be radioactively labelled by including ^{35}S in the medium, in the form of

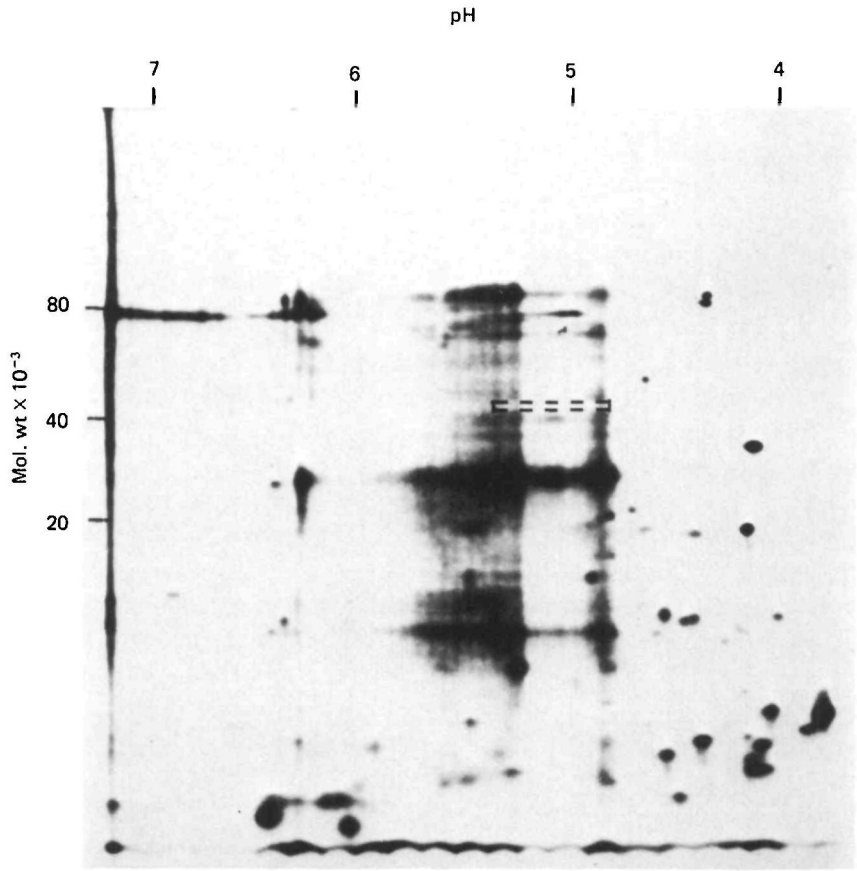

Fig. 9. Fluorograph of the 2-dimensional gel on which proteins of *A. longa* were separated. The sample applied to the gel contained unlabelled actin whose position was determined by Coomassie blue staining and is indicated by the broken lines.

sulphate, as the only source of sulphur. The proteins from cells labelled in this way were extracted and separated on 2-dimensional acrylamide gels (O'Farrell, 1975). A small amount of unlabelled actin from skeletal muscle and from brain was added to the extract. A fluorograph of such a gel (Fig. 9) prepared according to Laskey's description (Bonner & Laskey, 1974; Laskey & Mills, 1975) shows the location of radioactive peptides, with the position of actin (determined by staining with Coomassie blue) indicated. Very little radioactivity is present in this area. A quantitative estimate was obtained by cutting out the Coomassie-stained region of a duplicate gel

and measuring the included radioactivity by scintillation counting. The actin band consisted of a major spot at an isoelectric point of about 5·4 and a much weaker smear extending from 5·3 to about 4·9, all at a molecular weight of 43000. Assuming all of the counts in this area were from actin (which contains 21 sulphur atoms; Elzinga, Collins, Kuehl & Adelstein, 1973; Elzinga & Lu, 1976), it is calculated that each cell contained less than 6000 actin molecules. This is sufficient to make about 15 μm of F-actin filament. *A. longa* is about 40 μm long at rest, and can quickly change its length by a factor of 2. Thus euglenoid movement is probably not based on an actomyosin system.

DISCUSSION

The information available concerning euglenoid movement is far from complete, but it is possible at this point to make a reasonable hypothesis about how these unicellular algae control their shape. The observations that contribute to the hypothesis will first be summarized, specifically for *E. gracilis* (and *A. longa*, derived from *E. gracilis* by loss of chloroplasts). (1) The cells are capable of changing their shape rapidly; a halving of overall length may occur in less than 1 s. (2) Regions of the surface separated by 5 or 10 μm often show different rates and directions of movement. (3) The extreme shapes of the cell, fully elongated and round, can be induced by manipulating the intracellular calcium level. (4) The surface of the cell is constructed of a number of ridges beneath each of which is an essentially identical set of structures including a microtubule complex, fibrillar protein, and a channel of endoplasmic reticulum. (5) An intracellular pool of calcium exists, at least part of which is contained within the subpellicular endoplasmic reticulum. (6) A crude microsomal fraction can be prepared, which exhibits ATP-dependent Ca^{2+} transport, and which releases bound Ca^{2+} when exposed to an agent that causes shape changes in intact cells (caffeine). (7) The pellicle complex has a tightly associated ATPase activity.

An explanation for the cell's behaviour must propose a few further items that have not yet been proven. The repeating unit of the pellicle complex, fibrils, microtubules and associated structures, and endoplasmic reticulum is presumed to be an ATP-driven 'motor unit', capable of independent force generation. Activation of each unit tends to deform it from a nearly straight ridge (resting state) into a helix of a few turns (disk-shaped cell). This activation is normally mediated by calcium released from the channel of endoplasmic reticulum below each ridge. This reticulum also actively sequesters calcium so that the spread of any local calcium that is released is limited to a small area, no more than a few motor units away from the source. The effect of caffeine is to release Ca^{2+} from all the endoplasmic reticulum, causing a more or less symmetrical contraction. Similarly, raising the entire cellular Ca^{2+} level with ionophores activates all motor units equally, giving a symmetrical result. In normal motion, however, the independent activation of small areas would be accomplished by rapid sequestering of any Ca^{2+} that diffused away from the activated region by the endoplasmic reticulum channels of neighbouring unactivated units.

This explanation leaves out many important aspects of euglenoid movement about

which we have too little information to speculate. There must be some form of signal between the external environment and the endoplasmic reticulum that would normally trigger local motion. In some way the ambient light intensity must be communicated to the endoplasmic reticulum of *E. gracilis*. However, the most important omission from the hypothesis is the identity of the force-producing structure within the cell, and the nature of its motion. The presence of an ATP hydrolysis site in the region of the microtubule complex is suggestive, but further investigation is needed before a more complete explanation can be proposed.

Whether or not these microtubules are part of the motile apparatus, their tight association with the pellicle throughout the isolation procedure is an interesting phenomenon in itself. There have been several studies of the components that couple microfilaments to membranes (Bretscher & Weber, 1979; Goldman, Talian, Goldman & Chajnacki, 1979), and one might expect analogous proteins to exist for microtubules. The pellicle of one of the more easily cultured Euglenophyceae may be a useful source of such a protein.

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