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ISOLATED FLAGELLAR APPARATUS OF *CHLAMYDOMONAS*: CHARACTERIZATION OF FORWARD SWIMMING AND ALTERATION OF WAVEFORM AND REVERSAL OF MOTION BY CALCIUM IONS *IN VITRO*

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SUMMARY

The control of flagellar activity in the biflagellate green alga, *Chlamydomonas reinhardtii* was investigated by the *in vitro* reactivation of the isolated flagellar apparatus (the 2 flagella attached to their respective basal bodies plus accessory structures). The waveform and beat frequency of the isolated apparatus in the presence of 1 mM adenosine triphosphate (ATP) were comparable to those recorded for living cells. Equimolar concentrations of adenosine diphosphate (ADP) could be substituted for ATP with little change in beat frequency and no apparent change in waveform, suggesting that the latter is converted to ATP by axonemal adenylate kinase. No reactivation occurred in adenosine monophosphate (AMP). Beat frequencies in cytidine, guanosine and uridine triphosphates (CTP, GTP and UTP) were approximately 10% that obtained in ATP. Reactivation was optimal over a broad pH range between pH 6·4 and pH 8·9 in both ATP and ADP.

Isolated flagellar apparatus could be induced to change from forward to reverse motion *in vitro* by manipulation of exogenous calcium ions. The 2 types of motion were directly comparable to recorded responses of living cells. Forward swimming occurred at levels of calcium below 10^{-6} M, the isolated apparatus changing to backward motion above this level. Motility was inhibited at concentrations above 10^{-3} M. The threshold for reversal of motion by calcium was lowered to 10^{-7}M when the flagellar membranes were solubilized with detergent, indicating that the flagellar membranes are involved in the regulation of the level of calcium within the axoneme. The reversal of motion by calcium was itself freely reversible. The relationship of these observations to the known tactic responses of *Chlamydomonas* is discussed.

INTRODUCTION

Locomotion in many simple eukaryotes is achieved by the coordinated activity of from two to several thousand cilia. Transient changes in this pattern of ciliary beating are responsible for the directed movement of the cell in response to external stimuli and hence determine its characteristic tactic or behavioural responses. The mechanism of ciliary coordination is now fairly well understood and is thought to derive most commonly from viscoelastic coupling between autonomously beating oscillators through the fluid medium (Machemer, 1974), although for the ciliary

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comb plates of certain ctenophores, coordination appears to be mediated through some kind of internal conduction event (Tamm, 1973). A mechanism different from both of these may occur in the case of the unicellular green alga, *Chlamydomonas reinhardtii* (Ringo, 1967; Hyams & Borisy, 1975*a*). Although they are commonly referred to as flagella, the motile appendages of *Chlamydomonas* generate a pattern of beating which is typical of true cilia (Ringo, 1966; Hyams & Borisy, 1975*a*). Recently we were able to demonstrate that the coordinated beating of these organelles was a property intrinsic to the flagellar apparatus itself. Flagellar apparatus isolated from the cell were shown to maintain their characteristic motion if supplied with ATP in a suitable ionic environment (Hyams & Borisy, 1975*a*). In this paper we seek to analyse further the properties of ciliary coordination in *Chlamydomonas* by means of this unique *in vitro* system. Further, we wish to see whether we can identify factors which regulate the activity of these isolated flagella, with the aim of understanding some of the complex behavioural responses of this organism, notably with respect to light. Since a considerable body of evidence now suggests that calcium ions regulate the form and direction of ciliary and flagellar beating and that influxes of calcium ions mediate tactic behaviour (Naitoh & Kaneko, 1974; Brokaw, Josslin & Bobrow, 1974; Holwill & McGregor, 1975, 1976), we have investigated, in particular, the effect of calcium upon the reactivation of the isolated flagellar apparatus.

Preliminary accounts of some of this work have appeared elsewhere (Hyams & Borisy, 1974, 1975*b*).

MATERIALS AND METHODS

Flagellar apparatus were isolated from the wall-less mutant of *C. reinhardtii*, CW 92 (Hyams & Davies, 1972). Synchronous cultures of this mutant strain were harvested, washed and resuspended in isolation solution as described previously (Hyams & Borisy, 1975*a*). Experiments to determine the form and frequency of flagellar beating were performed using what will be referred to as 'standard reactivation conditions'. In these cases the composition of the isolation solution was as follows: 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid), 5 mM $MgSO_4$, 1 mM DTT (dithiothreitol), 0·5 mM EDTA (ethylenediaminetetra-acetic acid), 25 mM KCl, pH 7·0. Suspensions of flagellar apparatus in isolation solution were reactivated by mixing with an equal volume of reactivation solution as follows: 30 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 0·5 mM EDTA, 25 mM KCl, 2 mM ATP, pH 7·0. In those experiments which required the removal of the flagellar membrane, 0·1% Nonidet P-40 (Shell Chemicals) was included in the reactivation solution.

To determine the effects of calcium upon the form and frequency of flagellar movement, the isolation and reactivation solutions employed were those of Brokaw *et al.* (1974). The isolation solution contained 2 mM Tris, 2 mM $MgSO_4$, 2 mM DTT, 0·5 mM EDTA, 150 mM KCl, pH 8·0. Aliquots (0·5 ml) of a suspension of flagellar apparatus in this solution were centrifuged at 19000*g* (Sorvall rotor No. SS-34) for 20 min and the pellet gently resuspended in 0·5 ml of reactivation solution. This comprised 20 mM Tris, 2 mM DTT, 150 mM KCl and varying levels of $MgSO_4$, ATP and $CaCl_2$ to give a $MgATP^{2-}$ concentration of 1 mM, a Mg^{2+} concentration of 0·5 mM and a range of values of free calcium between 10^{-3} and 10^{-9} M (Brokaw *et al.* 1974). Demembranation of the isolated apparatus, where required, was achieved as above.

Qualitative examination of reactivation in the presence of calcium was performed by perfusing suspensions of flagellar apparatus in isolation solution with the appropriate reactivation solution. The perfusion chamber consisted of a glass slide and coverslip separated by 2 parallel streaks of silicone grease. A drop of suspension of flagellar apparatus was placed

between the lines of grease, and the coverslip placed over it so that 2 of its edges rested on the grease, thus forming a chamber which was open on two sides. A drop of reactivation solution was placed at one open end of the chamber and a wick of filter paper at the other to draw the solution into the chamber. In this way the ionic environment inside the chamber could be easily and quickly manipulated and any change in the waveform of the reactivating flagella recorded.

Reactivation, either in standard conditions or when calcium was present in the medium, was observed and recorded using a Zeiss Universal or WL microscope with a Neofluar 40/0·75 phase-contrast objective. Illumination for photography was provided by a xenon flash tube (Strobex Model 136, Chadwick-Helmuth Co., Monrovia, California) calibrated by means of an oscilloscope (Tetronix, Model 547). Both flash rate and exposure time were adjusted to obtain multiple images of a single flagellar apparatus per frame. Photography was on Tri-X film with Mikrodol developer. Beat frequencies of both living cells and isolated flagella were determined by adjusting the flash rate of the strobe until the motion of the flagella had apparently stopped, i.e. when flash rate equalled beat frequency. To facilitate measurements, only cells or flagellar apparatus which had become attached to the microscope slide in such a way that the flagella were still free to beat freely were recorded.

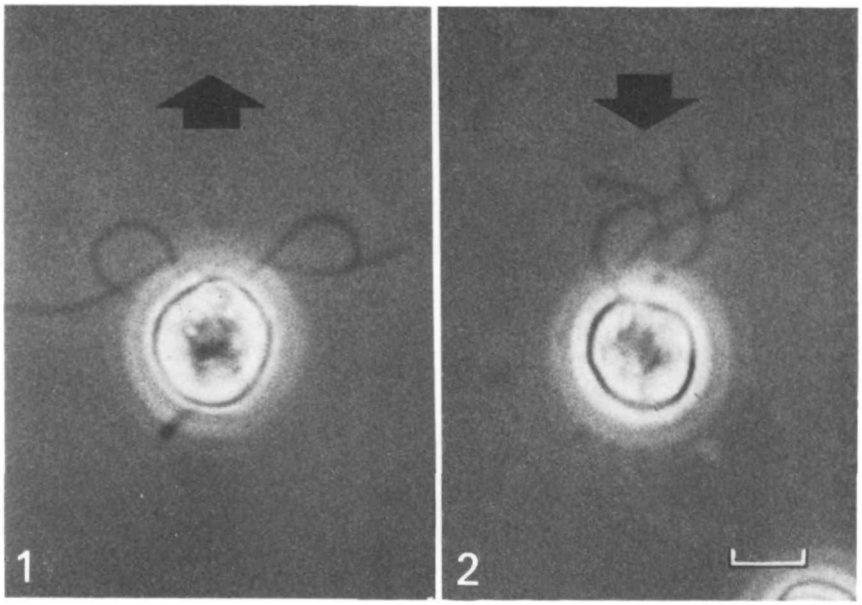
For examination by electron microscopy, preparations of intact flagellar apparatus were negatively stained with uranyl acetate and photographed with the objective aperture of the microscope removed to reduce contrast. Demembranated apparatus were first fixed with glutaraldehyde prior to staining with phosphotungstic acid.

RESULTS

Movement of living cells

Observation of populations of the wall-less mutant of *C. reinhardtii*, CW 92, revealed that individual cells frequently became attached to the microscope slide in such a way that the flagella were free to beat whilst the cell remained in a fixed position. Such cells offered a more convenient means of recording the patterns of flagellar movement than cells swimming freely and were used for the analysis of flagellar movement *in vivo*. The predominant pattern of beating seen in these tethered cells was the coordinated, ciliary type of beating characteristic of forward swimming in this organism (Ringo, 1967; Hyams & Borisy, 1975*a*). As with true cilia, the beat cycle of *Chlamydomonas* during forward swimming was biphasic, consisting of effective and recovery strokes. In the effective stroke, the flagella are held rigid and, bending only at their bases, are swept back past the cell. In the recovery stroke a wave is produced at the base of each flagellum and is propagated to the tip. The fact that one can obtain images of both phases of the beat cycle in the same photograph (Fig. 1) indicates that both occur in the same plane. The beat frequency of the flagella during forward swimming was determined to be 25 ± 5 Hz.

Occasionally the forward pattern of beating was disturbed, the flagella being thrown forward (Fig. 2) to beat vigorously for about 1 s before forward swimming was resumed. In free-swimming cells it was observed that this change in the pattern of beating caused the cell to *reverse* before proceeding in a new direction. Analysis of this reverse swimming was hampered by its short duration and irregular appearance but the few micrographs which were obtained showed coordinated waves passing out of phase to the distal end of the flagella (Fig. 2). Unlike the bend propagated during recovery stroke of forward swimming, these waves showed both a principal and a reverse bend and were similar to the motion of true flagella (Sleigh, 1974).



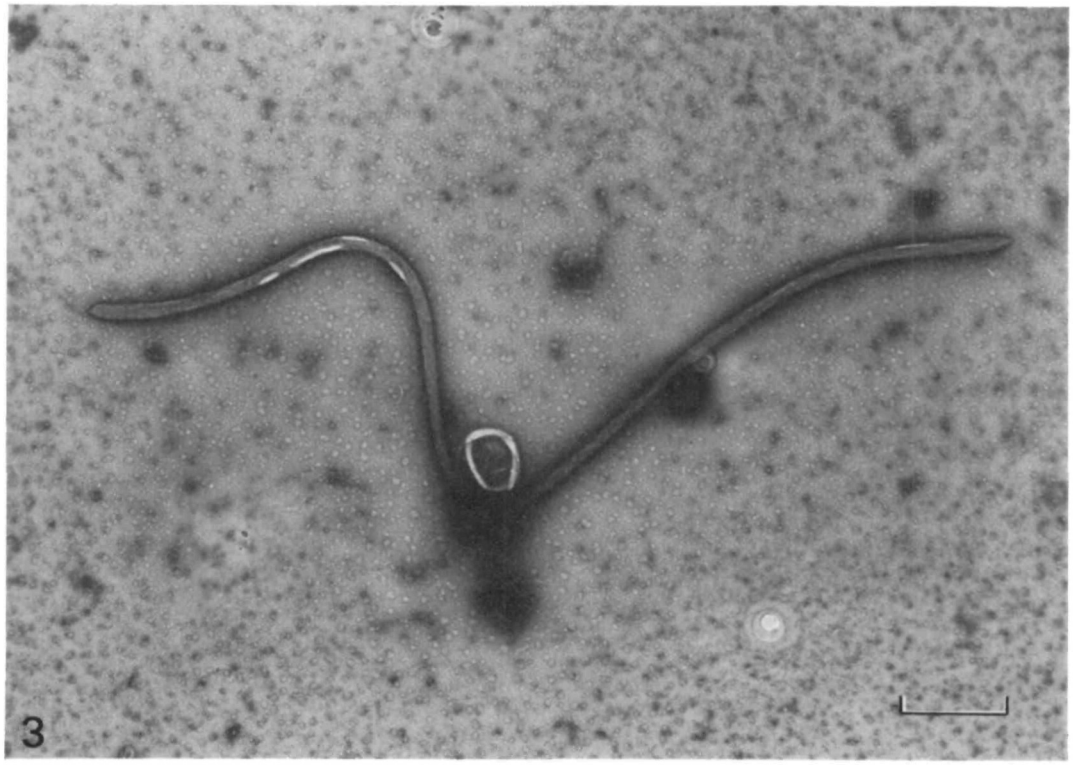
Figs. 1, 2. Flagellar motion in *Chlamydomonas reinhardtii*. The unicellular, biflagellate green alga, *C. reinhardtii*, swims predominantly forward (i.e. anterior end first) but reverses direction for short distances in response to an appropriate stimulus. Both forward and backward swimming are characterized by a distinctive pattern of flagellar beating, each of which is illustrated in these double-exposure micrographs. Fig. 1 shows both the effective and recovery components of the ciliary mode of beating associated with forward motion whilst Fig. 2 illustrates that backward swimming is effected by the propagation of undulatory waves more typically associated with flagella. The direction of movement in each case is indicated by the arrows. Phase optics, flash rate 31 Hz, exposure time 1/15 s, scale marker 5 μm.

Isolation and characterization of the flagellar apparatus

Before proceeding to the structure and movement of the isolated flagellar apparatus it is perhaps worth saying a few words about the nature of our isolation procedure. Wild type *Chlamydomonas* is enclosed by a multilayered cell wall which is extremely resistant to disruption by chemical or other methods (Roberts, Gurney-Smith & Hills, 1972). For this reason we chose to work with one of a large collection of mutant strains which are essentially genetic protoplasts, cells which are blocked in various aspects of cell wall biogenesis but yet retain normal growth and motile properties

Fig. 3. Whole-mount electron micrograph of isolated flagellar apparatus. The V configuration and the waveform of the flagella are evident. The axonemes remained enclosed by the flagellar membrane which terminate in the region of the basal bodies. The objective aperture of the microscope was removed to reduce contrast. Scale marker 2 μm.

Fig. 4. Basal region of the isolated flagellar apparatus. The flagellar membrane was removed with detergent revealing the microtubules of the axoneme (*a*). The 2 basal bodies form an obvious V and are connected by the striated fibre (*sf*). The transition region (*t*) of the basal body is also clearly visible. Fragments of microtubules forming the system of cytoplasmic rootlets (*r*) remain attached to the apparatus. This micrograph was generously provided by R. R. Gould. Scale bar 0·3 μm.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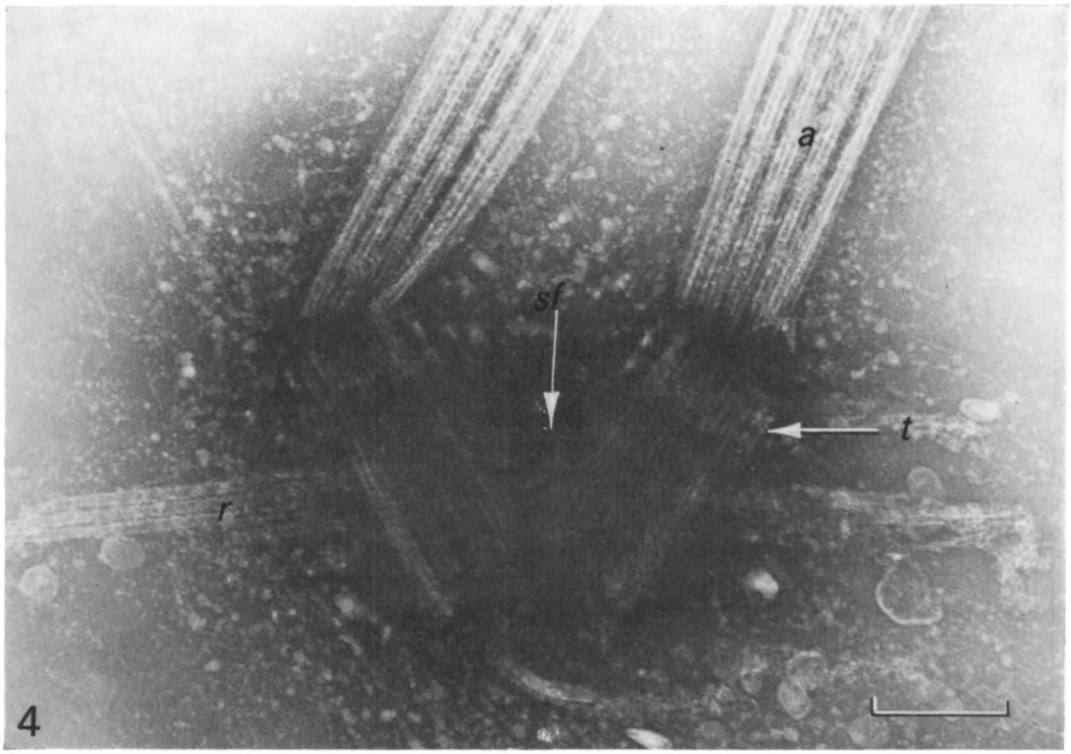
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(Hyams & Davies, 1972). Populations of one such mutant, CW 92, when harvested from the growth medium and resuspended in isolation solution were found to liberate their flagellar apparatus spontaneously. The yield however was low; only some 3% of the culture released their flagellar apparatus intact, whilst up to 30% of the cells shed single flagella (Hyams & Borisy, 1975*a*).

The reason why even a proportion of these cells should detach their flagella under these conditions is not known. Examination of cells synchronized by a light-dark regime throughout the vegetative cycle revealed that the maximum yield of flagellar apparatus was obtained shortly after the cells had entered the light period, the point at which cytokinesis is complete. The membrane of cells which are entering a period of rapid growth is therefore apparently especially sensitive to the trauma of resuspension in a solution of low ionic strength although the reason for this is not immediately obvious.

The yield of flagellar apparatus from these wall-less cells can be readily increased to 100% by lowering the magnesium ion concentration of the isolation solution and adding 0·1–0·5% Nonidet P-40 (Gould, 1975; our unpublished observations). So far, however, it has not been possible to reactivate flagellar apparatus isolated in this way. The lack of reactivation may have been due to some inhibitory factor liberated by the lysis of the cell population. That this is indeed the case has been shown by the addition of lysates from such experiments to flagellar apparatus prepared and reactivated under standard conditions, whereupon motility rapidly ceased. Consequently, we have contented ourselves with the low yield (3%) of reactivatable apparatus provided by our standard protocol and which is sufficient for the experiments reported below.

The isolated flagellar apparatus retained the V configuration characteristic of the apparatus *in situ* (Ringo, 1967; Fig. 3). The flagellar membranes apparently survived the isolation procedure and terminated just below the transition region of each flagellum, i.e. at the point where the flagellar membranes become contiguous with the cell membrane. However, to see details in such isolated structures by electron microscopy using negative staining proved difficult due to the dense accumulation of strain around the apparatus, particularly at the proximal end. To facilitate examination of the apparatus, its membrane was removed by detergent treatment. It was then possible to determine that each flagellum remained attached to its own basal body and that the 2 basal bodies were held at an angle of approximately 90° to each other (Fig. 4). The single striated fibre which connects the mid-lateral faces of the basal bodies was always observed unless the accumulation of stain prevented visualization of this region. The smaller fibres connecting the proximal ends of the basal bodies were also seen. Probasal bodies (Gould, 1975), although not evident in this figure were often observed attached to the basal bodies of the isolated apparatus. It is worth noting in Fig. 4 that the axonemal shaft apparently has been broken from the basal body just above the transitional zone. This weak point in the flagellar apparatus probably accounts for the low yield of V's and high percentage of single flagella noted earlier. For an extensive description of the flagellar apparatus of *C. reinhardtii* see Ringo (1967).

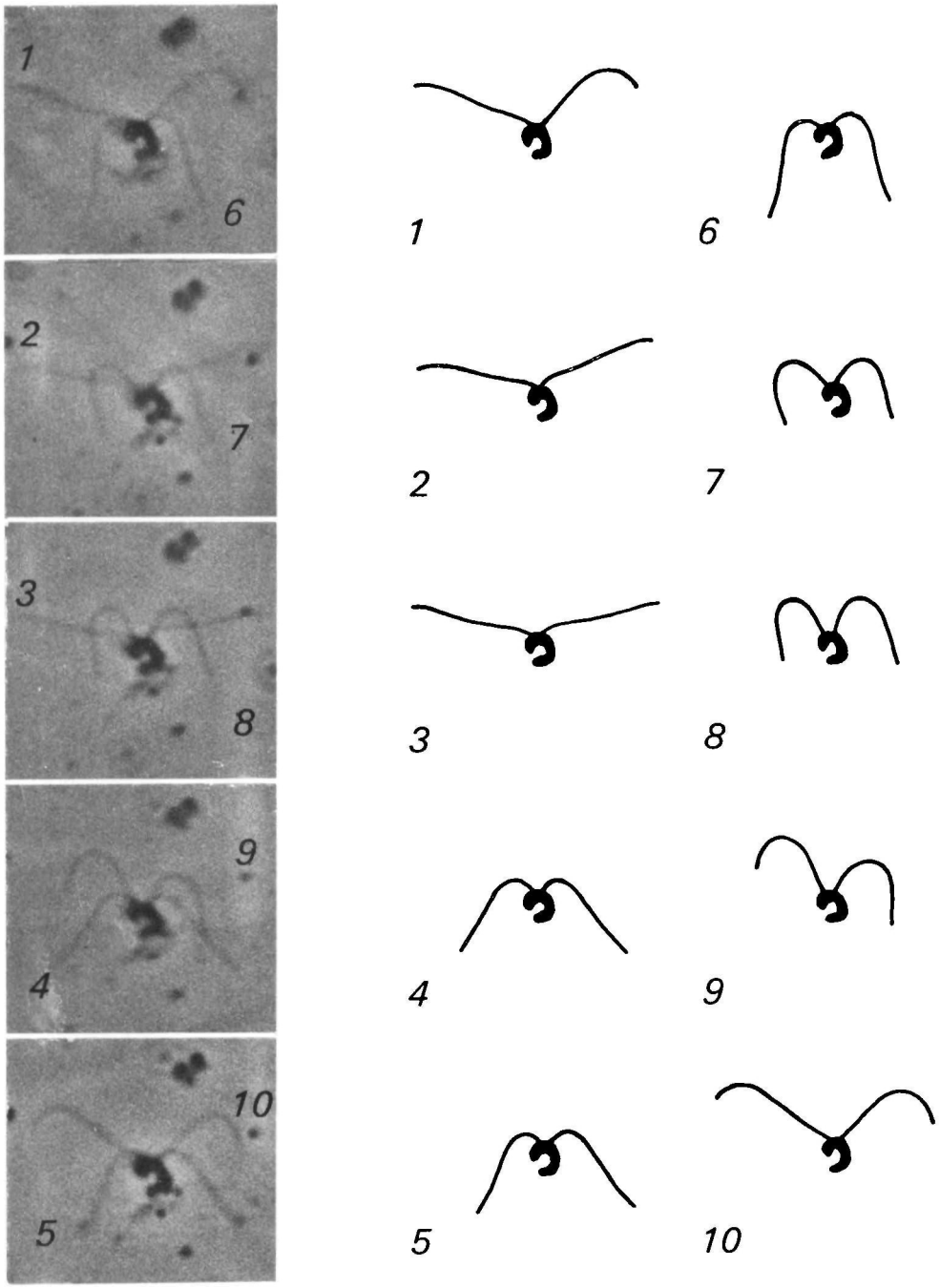

Fig. 5. Reactivation of the isolated flagellar apparatus. A double-exposure sequence of a single apparatus is shown in the left-hand panel. The apparatus was tethered to the glass slide by a piece of debris. For clarity, the individual images have been traced to show the effective (positions 1–5) and recovery (positions 6–10) phases of the ciliary beat. Phase optics; flash rate 31 Hz, exposure time 1/15 s.

Movement of the isolated flagellar apparatus

Upon the addition of ATP to 1 mM in standard reactivation conditions, more than 90% of the isolated apparatus immediately began to swim (Hyams & Borisy, 1975*a*). Swimming was always *forwards* (taking the basal bodies to be at the posterior of the apparatus), the isolated apparatus being seen either to swim across the microscope field whilst remaining in the same plane, or to tumble 'head over heels' in and out of the plane of focus. Occasionally apparatus became attached by their bases to the microscope slide and remained beating in a fixed position. A sequence of one such apparatus is shown in Fig. 5 to illustrate the nature of the flagellar motion. From the traces of the individual images of the flagella (shown in the 2 right-hand panels of Fig. 5) it can be seen that the beat cycle of the isolated apparatus is, qualitatively at least, indistinguishable from the ciliary motion of the living cell. The biphasic nature of the motion is evident and the effective (positions *1–5*) and recovery (positions *6–10*) components are easily identified. We have observed an individual apparatus maintain this type of motion for 2–3 h without any apparent alteration in the beat form. Flagellar apparatus which have been kept for up to 24 h at 4 °C in isolation solution prior to reactivation, reactivate with approximately 80% of the efficiency of samples reactivated immediately following isolation and show no obvious change in the nature of their motion.

To determine whether the integrity of the flagellar membranes was essential for the reactivation of the isolated apparatus the latter were reactivated in the presence of the detergent Nonidet. Concentrations of detergent between 0·01 and 1·0% had little effect on the efficiency of reactivation of the isolated apparatus (not shown) or upon the nature of its motion, although it was clear from the loss of refractility of the structure, as seen under phase-contrast microscopy, and from observation by electron microscopy (Fig. 4) that the membranes were solubilized at concentrations of detergent above 0·05%. Further evidence to this point was provided by observation of single flagella, detached from their basal bodies during the isolation procedure (Hyams & Borisy, 1975*a*). In the absence of detergent in the reactivation solution less than 1% of these single flagella showed any form of motion. In the presence of 0·1% Nonidet, however, 30–40% of these single flagella have been observed to perform the type of motions previously described for isolated, demembranated single flagella from *C. reinhardtii* (Allen & Borisy, 1974).

The large number of photographs of reactivating flagella apparatus taken during the analysis of the form of the beat also allowed us to determine the timing of the various events of the beat cycle. Since during a beat cycle the shaft of each flagellum moves and returns through an arc of approximately 120°, it was possible to divide the cycle into 5 easily identifiable and equal stages each of $(2 \times 120°)/5 = 48°$. These were designated positions *1–5* and are shown schematically by the shaded images across the top of Fig. 6. Positions *1* and *2* represent the early and late portions of the effective stroke, positions *4* and *5* the corresponding portions of the recovery stroke, and position *3* the point of changeover between the two. Photomicrographs of reactivating apparatus stopped in motion by the flashing of the strobe were scored

as to which of these 5 positions they corresponded. Since the time a flagellum spends in each position of the cycle is inversely related to the speed at which it is travelling, by counting the numbers of flagella seen in each position we have a measure of the timing of the beat cycle. The results of this analysis are shown by the open histogram in Fig. 6. It is clear from this analysis that the speed at which the flagella travel shows a rhythmic variation through the beat cycle, being fastest through the effective stroke and slower through the recovery stroke. For comparison, a similar

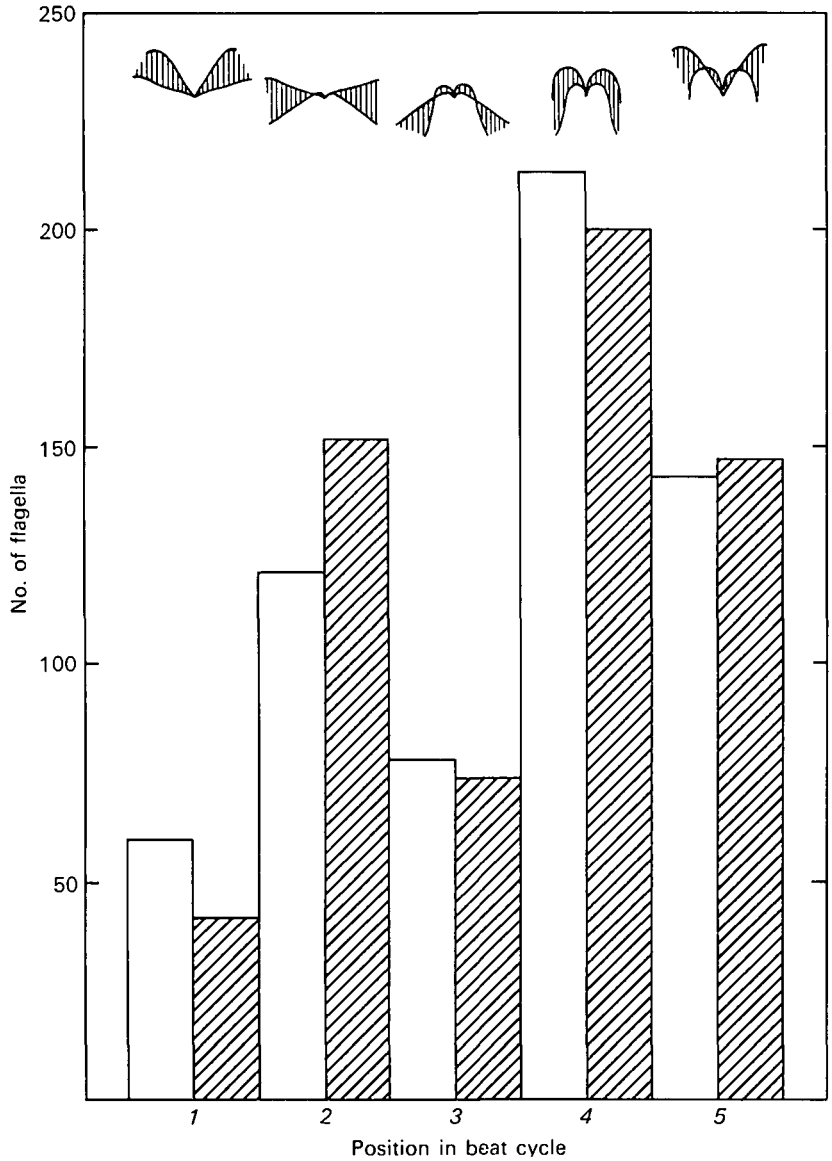

Fig. 6. Timing of the phases of the beat cycle. The beat cycle was divided into 5 phases and analysed as described in the text. Data are shown for flagellar beating on isolated apparatus (open bars) and living cells (hatched bars).

analysis performed on living *Chlamydomonas* is shown in the shaded histogram in Fig. 6. If one compares the beat patterns of living and reactivated flagella it can be seen that not only do the isolated flagella retain the characteristic pattern of beating of the living cell but also the timing of the events of the beat cycle are virtually indistinguishable from the *in vivo* pattern.

Conditions of reactivation

The biochemical parameters regulating the motility of *single*, demembranated axonemes are now reasonably well understood for several *in vitro* systems (Gibbons & Gibbons, 1972) including *Chlamydomonas* (Allen & Borisy, 1974). However, since the coordinated beating of 2 flagella which retain their membranes might present different problems of regulation from single axonemes, some of the biochemical variables of reactivation were investigated.

The percentage reactivation seen in preparations of isolated flagellar apparatus was a function of the concentration of ATP added. Maximum levels of reactivation (> 90%) were observed at concentrations of ATP above 0·1 mM; below this level reactivation declined sharply and no movement was observed below 0·01 mM ATP. A similar response was observed if ADP was substituted for ATP in the reactivation solution, maximum reactivation (again > 90%) being seen above 0·1 mM ADP, below which reactivation declined sharply with no movement seen at 0·05 mM ADP. No reactivation at all was seen if the adenine nucleoside in the reactivation solution was supplied in the form of AMP. Since ATP and ADP appeared indistinguishable in inducing the reactivation of the isolated flagellar apparatus it was of interest to determine whether the beat frequencies obtained in the presence of the 2 adenine nucleosides were also comparable. Measurements of the beat frequencies of 20 flagellar apparatus in 1 mM solutions of either ATP or ADP gave values of 18 ± 3 Hz for ATP and 17 ± 3 Hz for ADP.

To determine whether other nucleotide triphosphates could substitute for ATP in inducing motility in the isolated apparatus, a further experiment was performed in which apparatus were exposed to ATP and to CTP, GTP and UTP, all at a concentration of 1 mM. The results indicated that the coordinated movement of the isolated flagellar apparatus is not specific for ATP. Levels of reactivation seen with CTP (90%), UTP (93%) were comparable to those with ATP (97%), although lower levels were observed in the presence of GTP (64%). However, when the beat frequencies of the flagellar apparatus in the presence of the various nucleotide triphosphates were compared it was seen that only in the presence of ATP did the beat frequency approach that recorded for living cells (25 ± 5 Hz). In the presence of CTP, GTP or UTP the beat frequencies measured were only about one tenth of the rate recorded for ATP, although the nature of the beat was apparently unchanged.

The pH range of reactivation was also investigated in the presence of both ATP and ADP. Six buffers (citrate, MES, PIPES, HEPES, Tris, phosphate) were used in limited, overlapping ranges to provide a wide pH spectrum. Motility with either substrate showed a broad plateau between pH 6·4 and 8·9, with small optima at

pH 6·5 and 8·7. Either side of this range the per cent reactivation declined sharply, being somewhat less abrupt on the acidic side of the scale, with no reactivation seen below pH 5·1 or above pH 9·5. No specificity for any of the buffers employed was noted.

Effect of calcium on flagellar waveform and direction of movement

In order to obtain conditions in which the level of free calcium ions in the reactivation solutions could be precisely controlled, investigation of flagellar movement in the presence of calcium was based largely on the data of Brokaw *et al.* (1974;

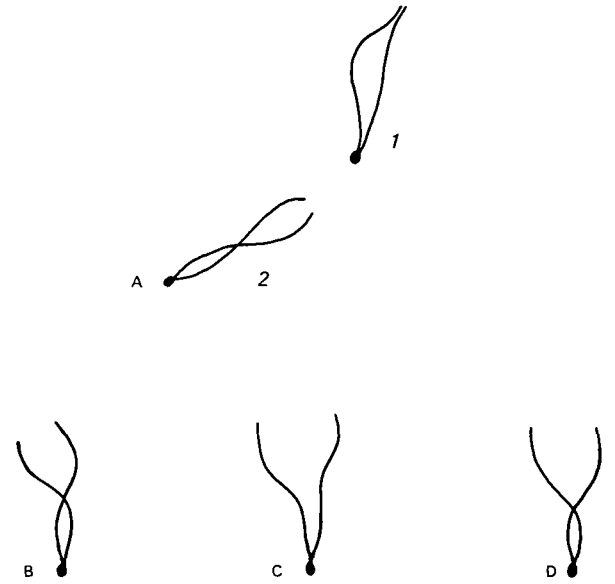

Fig. 7. The reversal of flagellar motion by calcium ions. The addition of calcium ions to the reactivation solutions caused a change in both the waveform and direction of beating of the isolated flagellar apparatus. The direction of swimming is now *backwards* (i.e. basal bodies first) and the beat pattern resembles the flagellar-like motion described for the backward motion of the cell. A, 2 exposures (1, 2) on a single frame illustrating the direction of movement. B–D, additional exposures of the same apparatus showing other aspects of the flagellar waveform.

Materials and methods). Isolated flagellar apparatus resuspended in solutions containing 10^{-6} M calcium or less, immediately commenced the frenetic, coordinated beating described previously (Fig. 5). Upon raising the calcium concentration to 10^{-5} M however, a dramatic alteration was observed both in the form and frequency of flagellar beating. Under these conditions the isolated apparatus was observed to swim *backwards* (basal bodies first) or in other words to have changed their direction of movement in response to a defined concentration of calcium ions.

The nature of the waveform during reverse swimming was markedly different for the coordinated ciliary mode recorded for forward movement. The flagella were no longer held at a 90° angle to each other (the 'V' configuration) but instead were held

virtually parallel, with successive, coordinated, flagellar-like waves passing along each flagellum in a proximal-to-distal direction at a frequency of 24 ± 4 Hz (25 measurements; see Fig. 7). Although this is a higher frequency than recorded for forward swimming (but note that the reactivation conditions are not directly comparable) the backward swimming apparatus progressed across the microscope field at a much slower rate than the forward swimming apparatus and exhibited none of the frenetic behaviour displayed by the forward swimmers. Also, backward swimmers

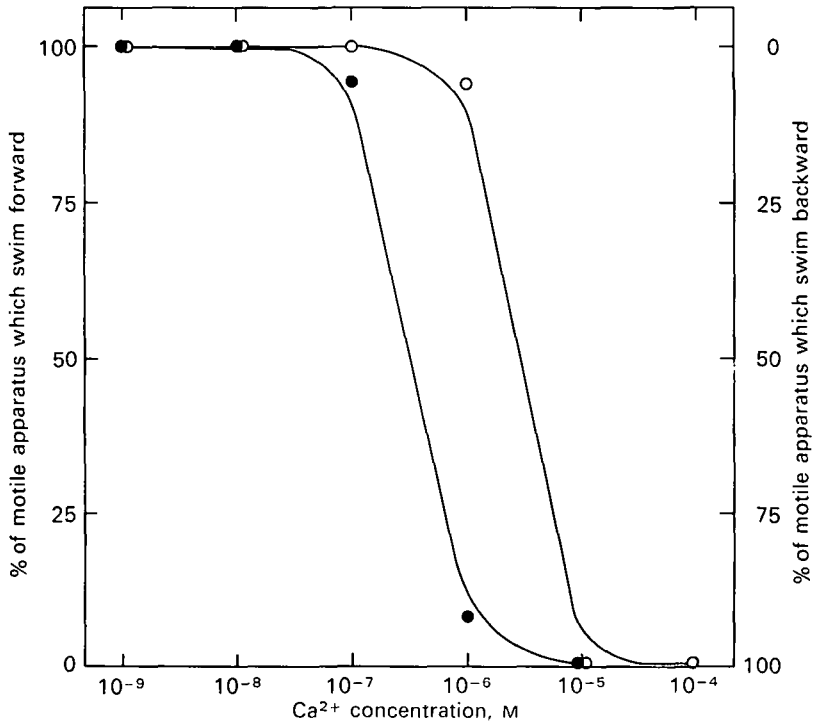

Fig. 8. Quantitation of the reversal of flagellar beating in response to calcium ions. Flagellar apparatus were reactivated in solutions containing buffered levels of Ca^{2+}. Apparatus in which the membranes were solubilized by detergent (●) showed a 10-fold lower threshold for reversal of swimming than those in which the membranes were intact (○).

did not perform the 'head-over-heels' tumbling motion typical of forward movement but swam in one plane, rotating slowly about the long axis of the apparatus, approximately 1 revolution per 2·5 s. If the level of calcium in the reactivation solution was raised to 10^{-3} M no movement of the flagellar apparatus was detected.

When 0·1 % Nonidet was added to the reactivation solution to remove the flagellar membranes, the same response to calcium was observed but at a lower concentration of calcium than if the membranes were present. In the absence of the flagellar membranes the apparatus swam *forwards* at calcium concentrations below 10^{-7} M, *backwards* if the calcium level was raised to 10^{-6} M and motility was completely inhibited by 10^{-4} M calcium. Thus the effect of removing the flagellar membranes

was to lower both the point at which the transition between the 2 types of motion occurred and the level of calcium which was inhibitory to reactivation by 10-fold. This response is shown graphically in Fig. 8.

The effect of calcium in reversing the direction of swimming of the isolated apparatus is in itself *completely reversible.* Flagellar apparatus were reactivated in 10^{-5} M calcium to induce backward swimming and placed in a perfusion chamber (Methods). A field was selected in which one or more reactivating apparatus had become attached to the microscope slide and the solution in the chamber replaced with 10^{-6} M calcium by perfusion. As the higher level of calcium reached the reactivating apparatus the flagellar-like waves ceased, the angle between the flagella widened (to form a V) and forward, ciliary beating was assumed. When the calcium concentration was once again lowered by perfusion of the 10^{-6} M solution, the

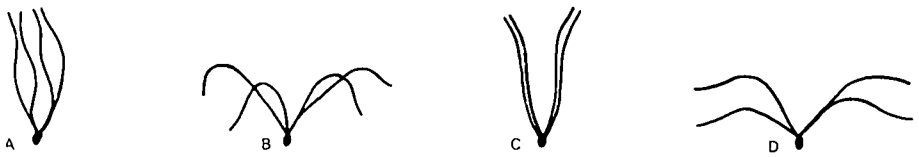

Fig. 9. The reversal of flagellar motion by calcium is itself reversible. By raising or lowering the concentration of Ca^{2+} ions across the threshold required for reversal, flagellar apparatus were induced to alternate between forward and backward motion. This sequence shows tracings of an individual apparatus. Concentrations of calcium in A, B, C, D were 10^{-5}, 10^{-6}, 10^{-5} and 10^{-6} M respectively. Multiple exposures of the apparatus were recorded.

ciliary beating ceased, the angle between the flagella closed and backward swimming was again resumed. A sequence of the transitions between backward and forward motion in a single flagellar apparatus is shown in Fig. 9. After 3 or 4 changes of calcium solution the beating of the flagella usually ceased but the *non-reactivating apparatus still responded to calcium* in that the angle between the flagella widened upon the removal of calcium and closed when the calcium was restored.

DISCUSSION

Flagellar motion in vivo and in vitro

Although a comprehensive description of the swimming action of *C. reinhardtii* has been presented previously by Ringo (1967) we considered it necessary to re-examine the problem using conditions which were directly comparable to those employed for recording the form and frequency of flagellar motion *in vitro.* His scheme derived from observation of wild-type cells immersed in viscous medium to reduce the beat frequency of the flagella to about 1 Hz, whilst we recorded cells of the wall-less line CW 92, beating at 25 times that rate. Nevertheless, our observations of the forward swimming of the organism agree extremely well with Ringo's description. We have used the terms 'effective stroke' and 'recovery stroke' in preference to Ringo's terminology of 'power' and 'return' strokes, since the former are consistent with previous descriptions of ciliary beating (Sleigh, 1974) and more

correctly reflect the hydrodynamic properties of this type of flagellar movement (Blake & Sleigh, 1974). Our description of backward swimming differs slightly from Ringo's in that although we both note the coordinated propagation of waves toward the distal end of the flagella, our observations suggest that the waves are directly out of phase, whereas Ringo shows an in-phase relationship. This aspect of motility in *Chlamydomonas* remains too poorly documented to reconcile this difference at present, although one might expect that adjacent flagella would beat in phase due to visco-elastic coupling through the medium (Machemer, 1974).

The movement of the isolated flagellar apparatus under standard reactivation conditions is at least qualitatively indistinguishable from the motion of the flagella on the living cell. The form of the beat and timing of the beat cycle of the flagellar apparatus are directly comparable to the swimming action of *C. reinhardtii in vivo*. The beat frequency of the flagellar apparatus is about 30% less than that seen on the cell, although the possibility that a higher beat frequency might be obtained by raising the $MgATP^{2-}$ concentration in the reactivation solution has not been investigated.

Isolated flagellar apparatus are able to reactivate without prior removal of the flagellar membranes, since the latter terminate mid-way along the length of the basal bodies, leaving their proximal end open to the free diffusion of ATP into the flagellar shafts, a mechanism which presumably reflects the *in vivo* situation. Single flagella, detached from their basal bodies during the isolation procedure, do not reactivate unless detergent is added to the reactivation solution to remove the flagellar membranes, indicating that the membrane reseals over the exposed proximal end of the detached axoneme and renders it inaccessible to ATP. That the flagellar membranes play no role in the form or coordination of flagellar activity is shown by the observation that the inclusion of detergent in the reactivation solution affects neither of these parameters.

Regulation of forward swimming in vitro

We approached the investigation of the parameters affecting the motility of the isolated flagellar apparatus during forward swimming to determine whether structures intrinsic to the apparatus other than the axonemes themselves played any role in this process. Maximum yields of reactivating flagellar apparatus were obtained in solutions containing ATP or ADP at concentrations above 0·1 mM. This level of ATP is comparable to that required for the reactivation of flagella from other organisms and is consistent with the estimated intracellular concentration of ATP in *C. reinhardtii* (Stavis, 1974). Reactivation in the presence of ADP indicates the conversion of ADP to ATP by axonemal adenylate kinase (Raff & Blum, 1968). This provides further evidence that the integrity of the membranes of the flagella apparatus survive the isolation procedure, since demembranated flagella reactivate only weakly in ADP, presumably because the ATP generated by the kinase is lost through diffusion (Brokaw & Gibbons, 1973). It has been proposed that adenylate kinase serves to maintain levels of ATP required for beating in the distal regions of long flagella where diffusion from the proximal end would be a limiting factor

(Raff & Blum, 1968). Here we have shown that even in the short flagella of *C. reinhardtii* (10–12 μm in length) sufficient adenylate kinase exists to maintain beat frequencies comparable to those seen *in vivo* using ADP as the sole source of adenine nucleotide. Since the patterns of beating seen in ADP are identical to those seen with ATP, these results may also suggest that the kinase is distributed along the length of the flagella.

The complete absence of reactivation in the presence of AMP indicates an energy dependence for motility upon a hydrolysable terminal phosphate group, a property expected of the ATPase protein dynein. Similarly the nucleotide specificity and pH dependence of motility in the presence of magnesium also resemble the dephosphorylation of ATP by dynein (Gibbons, 1966). Experiments using various nucleotide triphosphates showed that although comparable levels of reactivation were seen with CTP and UTP as with ATP (> 90%), albeit somewhat lower with GTP, the beat frequencies recorded in the presence of these other nucleotides were significantly lower than for ATP, the other nucleotide triphosphates being hydrolysed at one tenth its rate. The pH dependence of reactivation was similar to the enzymic activity of dynein. Reactivation showed a broad plateau between pH 6·4 and 8·9 with a small optima at pH 6·5 and 8·7. The activity of 30S dynein showed a similar plateau between pH 5·8 and 9·0, with optima at pH 6·2 and 8·5.

Our observations indicate that conditions favouring the coordinated forward swimming of *C. reinhardtii in vitro* parallel the specific properties of dynein (Gibbons, 1966). A similar observation has previously been reported for the *in vitro* motility of the sperm of the sea urchin, *Colobocentrotus* (Gibbons & Gibbons, 1972). However, in the latter case the reactivated flagella generate a typical flagellar beat, whilst the motion of the *Chlamydomonas* flagellar apparatus is clearly ciliary under virtually identical conditions.

Dependence of waveform and direction of movement upon calcium ions

Manipulation of the calcium ion concentration to which reactivated flagellar apparatus were exposed above or below a threshold of 10^{-6} M, induced a change in the motion of the flagella directly comparable to the forward and backward movement of living cells. When the flagellar membranes were removed by detergent treatment the threshold concentration of calcium at which reversal occurred was lowered to 10^{-7} M. This 10-fold difference in the response of flagellar apparatus with and without membranes was not due to a differential diffusion of calcium into the membranated apparatus, since the axonemes of the latter are in free contact with the external environment as demonstrated by their accessibility to exogenous ATP. Rather it indicates that the flagellar membranes play an active role in regulating the concentration of calcium within the axoneme, acting as a 'calcium pump' to maintain internal levels of calcium which are low relative to the external environment. Thus the the 'true' calcium concentration at which reversal occurs is 10^{-7} M as demonstrated by the detergent-treated apparatus. When membranated apparatus are placed in solutions containing 10^{-7} M calcium the flagellar membranes are able to maintain an intraaxonemal concentration below this level and reversal does not

occur. Only when the level of free calcium reaches 10^{-6} M is the membrane pump saturated, allowing the internal calcium concentration to rise above 10^{-7} M, initiating reversal.

The sliding tubule model of ciliary and flagellar motion predicts that interdoublet sliding using energy derived from the dephosphorylation of ATP by dynein, is converted into local bending by the cyclic attachment and detachment of radial spokes extending from the A tubule of the outer doublet to the projections forming the sheath around the central pair (Summers & Gibbons, 1973; Warner & Satir, 1974). As yet unexplained, however, is how one mechanism can account for 2 different types of motion (flagellar and ciliary) in apparently identical structures. Our observations on the interconversion of the 2 modes of beating of the flagella of *C. reinhardtii* demonstrate that both motions are inherent to a single 9+2 structure and that the beat form expressed depends upon the activation of a high-affinity calcium acceptor within the axoneme.

The nature of the calcium acceptor is at present unknown although the identification of a calcium-specific ATPase within the flagella of *C. reinhardtii* (Watanabe & Flavin, 1976) makes this a potential candidate. If this enzyme is indeed the acceptor, an understanding of its mechanism of action would be greatly facilitated by knowing its localization within the axoneme. That it is not associated with the central pair microtubules has been shown by the demonstration of normal enzyme levels in a mutant lacking this flagellar component (Watanabe & Flavin, 1976). Recent observations on *Paramecium* have indicated that calcium enters the axoneme only at its extreme proximal end via a ring of granule plaques which insert into the ciliary membrane in this region (Plattner, 1975). This is recognized by the deposition within the ciliary membrane, immediately distal to the basal bodies, of electron-dense deposits possibly consisting of calcium phosphate formed by localized ATPase activity (Plattner, 1975; Fisher, Kaneshiro & Peters, 1976). The corresponding region of the axoneme of *C. reinhardtii* is characterized by the presence of a unique type of interdoublet linkage distinct from the dynein side arms of the rest of the axoneme (Ringo, 1967). Whether these specialized linkages represent the calcium ATPase awaits the histochemical localization of the ATPase activity and calcium uptake in *Chlamydomonas* flagella, together with free-etch analysis of the flagellar membranes to look for an equivalent to the ciliary membrane plaques which have been proposed to represent calcium channels into the cilia of protozoa.

Another possibility for the calcium receptor is not enzymic but purely structural in nature and involves the proximal region of the flagellar apparatus. We have observed that isolated flagellar apparatus which have undergone several reversals of flagellar beating by manipulation of exogenous calcium gradually fail to continue beating. However, they still respond to calcium by an alteration in the angle subtended between the flagella, widening at low calcium and closing at higher calcium concentrations. This suggests the possibility that control of the waveform may be dissected experimentally from beating per se, an impression which is enforced by the report that glycerinated, non-motile *Paramecium* will reverse the orientation of their ciliary power stroke in the presence of Ca^{2+} and ATP (Naitoh, 1969).

Reversal of flagellar beating and behavioural responses of Chlamydomonas

The association of calcium regulation of flagellar activity with the tactic responses of microorganisms is now established for many organisms (Naitoh & Kaneko, 1972; Holwill & McGregor, 1976; Brokaw, 1963, 1974). The best characterized of these is the avoiding reaction of *Paramecium* where influx of calcium into the cell causes a reorientation of the cilia through 180° with corresponding reversal of direction (Eckert, 1972; Naitoh & Eckert, 1974). Although, unlike *Paramecium*, calcium-stimulated reversal of direction in *Chlamydomonas* is achieved by a change in waveform, it seems reasonable to conclude that reversal is a manifestation of a tactic response.

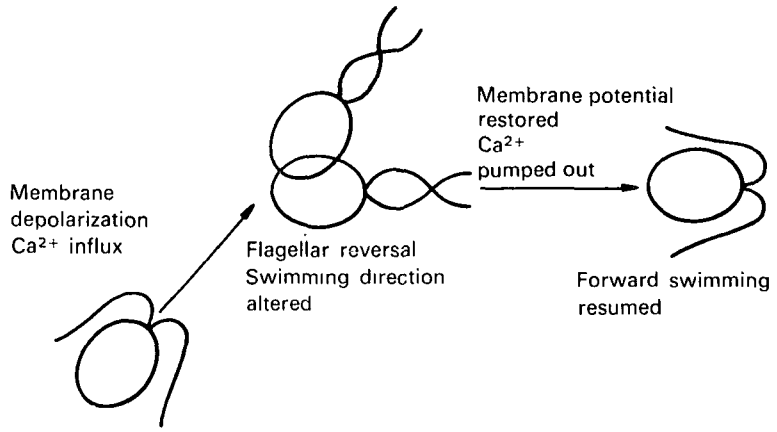

Fig. 10. Schematic representation of flagellar reversal in *C. reinhardtii*. Polarizing events involving the cell membrane are well documented in the case of flagellar reversal of *Paramecium* in response to calcium. Our scheme does not identify the inducer of the reversal response although *Chlamydomonas* is known to undergo such reactions in response to high light intensity and when encountering an obstacle.

The results from the reversal of flagellar beating by calcium *in vitro* have been assembled into a schematic representation of the behaviour of the living *Chlamydomonas* cell (Fig. 10). This scheme draws heavily on that already proposed for *Paramecium* (Naitoh & Eckert, 1974) and envisages that depolarization of the flagellar membranes in response to a stimulus allows the influx of calcium into the cell, thus inducing flagellar reversal. Once this change in swimming direction has taken the cell away from the stimulus, the resting potential of the flagellar membranes is restored, calcium is removed from the axonemes by the action of the flagellar membrane 'pump', and normal foward swimming is resumed.

The most likely inducer of flagellar reversal is light. This is shown directly by the experiments of Schmidt & Eckert (1976) who noted a similar change in flagellar waveform in living cells upon photostimulation in the presence of calcium ion concentrations in excess of 10^{-6} M. Phototaxis in *Chlamydomonas* is well documented (Feinleib & Curry, 1967; Stavis & Hirschberg, 1973) and has been shown to have a strict calcium requirement (Stavis & Hirschberg, 1973). Unfortunately, details of

flagellar waveform during reorientation of the cell towards light have not appeared in the literature, although it has been claimed that at the onset of photactive stimulation, cells exhibit a 'stop response' (Feinleib & Curry, 1971). Alternatively, the effect of calcium upon flagellar movement *in vitro* may represent an avoiding or 'shock' reaction seen in other green flagellates (Engelmann, 1882; Brokaw, 1963). Such behavioural patterns may be induced by light but may represent a general reaction to a wide spectrum of physical or chemical stimuli.

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