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MICROMANIPULATION OF THE FLAGELLUM OF *CRITHIDIA ONCOPELTI*

I. MECHANICAL EFFECTS*

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INTRODUCTION

Although considerable information about the fine structure of cilia and flagella has been accumulated in recent years (e.g. Warner, 1970; Linck, 1973), comparatively little new experimental work has been reported concerning the responses of flagella *in vivo* to external stimuli. Since theoretical models of the mechanisms which are responsible for flagellar activity are being developed (e.g. Brokaw, 1971; Lubliner & Blum, 1971; Miles & Holwill, 1971; Rikmenspoel, 1971), as much information as possible about the behaviour of the real system is required to test the predictions of the models. Two types of model have been discussed: a local contraction system and one which involves relative sliding of filaments within an organelle. At the present time most of the available experimental evidence supports the sliding filament model (see e.g. Brokaw, 1972) although some observations favour a local contraction system (Goldstein, Holwill & Silvester, 1970; Goldstein, 1972). The work reported by the latter authors involved localized damage to a flagellum inflicted by a laser microbeam; it is therefore of interest to study the responses of flagella to damage caused by other means so that comparisons can be made with the laser work. A convenient alternative is the application of micro-manipulator probes, and it is the purpose of this paper to present results of damaging and constricting the flagellum of *Crithidia oncopelti* by this technique.

MATERIAL AND METHODS

Cultures of *Crithidia oncopelti* were grown in the synthetic medium described by Newton (1957). After about 5 days the organisms were centrifuged, washed and resuspended in Ringer's solution, in which the cells had a strong tendency to adhere to the coverslip, a condition which proved useful for micromanipulation. The wave forms of flagella were identical in Ringer's solution and in culture medium. For experiments which involved description of the cell, the organisms were re-suspended in a solution containing 200 mol m^{-3} trishydroxymethylamine, 50 mol m^{-3} KCl, 4 mol m^{-3} $MgCl_2$ and 1 and 2 mol m^{-3} adenosine triphosphate (ATP), buffered with thioglycollic acid to pH 7·8.

Observations were carried out using a Meopta D microscope mounted on a Research Intruments TVC 500 Bio-Medical Micromanipulator. Probes were made from 2 mm

* NOTE. Throughout this paper concentration is expressed as mol m^{-3} to be consistent with the S.I. system of units. The relationship between this and the molar (M) unit is 1 mol m^{-3} = 1 mM.

bore capillary tubes which were first pulled in a Narishige (PE-2) vertical microelectrode-pulling machine and further extended and shaped in a de Fonbrune microforge. The final diameter of a microprobe tip was between 0·5 and 1·0 μm.

Organisms were suspended in a hanging drop on the underside of a coverslip and mounted in a specially designed oil chamber, similar to that described by de Fonbrune (1949). The size of the drop was controlled by means of a micropipette, and by reducing the drop diameter cells could be induced to adhere to the coverslip, a condition which was frequently convenient for micromanipulation. Observations were made using a $\times 45$ or $\times 100$ microscope objective. In general, micromanipulation slowed the flagellar movement sufficiently for it to be followed visually, but photographic records of certain phenomena were obtained using Zeiss and Exa cameras with FP 4 film.

RESULTS

Although the general features of the flagellar movement of *Crithidia oncopelti* have been described in detail elsewhere (Holwill, 1965), a summary will be given here of those details relevant to the present study. The flagellum has an average length and diameter of, respectively, 20 and 0·2 μm, and is attached to a cell body about 8 μm long and 3 μm in diameter which resembles a truncated cone with a small apex angle. Waves are usually propagated along the flagellum from its tip towards the base with average values of 14·4 μm, 2·4 μm and 17 Hz for the wavelength, amplitude and frequency respectively. Occasionally, and in particular when the flagellum touches an obstruction, the direction of wave propagation is reversed. A cell which propagates waves from flagellar tip to base swims with the flagellum preceding the body while when the direction of wave propagation is reversed the cell is pushed by the flagellum.

Effects of mechanical constriction and micro-dissection

If any region of the flagellum of a freely swimming cell which was propagating waves towards the base of its flagellum was touched briefly with a micro-probe, or if the fluid surrounding the flagellum was agitated, the direction of wave propagation was momentarily reversed. Soon after the stimulus was removed, the wave movement was once more observed to be from flagellar tip to base. For technical reasons micromanipulation techniques were used on cells which were adhering by their cell bodies to the coverslip. The cell bodies of such immobile organisms were often spherical in shape. Touching the flagellar tip of a cell restrained in this way frequently did not produce the wave reversal characteristic of the freely swimming organism.

When a probe was laid across the flagellum close to its tip and maintained in this position, bends were propagated from the base towards the tip. As the pressure exerted by the probe on the flagellum was increased, the frequency of bend propagation decreased while the amplitude of the wave increased. Further increase in the pressure applied by the probe caused wave propagation to be replaced by a local oscillation which had a frequency near that of normal flagellar movement. On removal of the probe, the flagellum executed the normal tip-to-base wave pattern after a short interval of time. The resumption of basally propagated waves was never observed to occur before the bend nearest the tip of the flagellum had been propagated to the tip. If the flagellar tip was dissected the flagellum propagated rapid asymmetric waves for

Table 1. *Summary of effects of constricting and dissecting the flagellum of* Crithidia oncopelti

Constriction or dissection	Region of flagellum manipulated	Behaviour of flagellum	
		Between body and probe	Between probe and tip
Constriction	Tip	b→ t	—
Dissection	Tip	b → t, t → b	—
Constriction	Base	—	b → t, t → b, l
Constriction	Middle	b → t, l	b → t, t → b, l
Dissection	Middle	b → t followed occasionally by t → b	Independent movement, b → t, t → b, *but* reversal not observed

b → t, Wave propagation from base to tip. t → b, Wave propagation from tip to base. l, Local oscillation.

a short time before it became straight and quiescent or executed slow oscillations. After a further period, the flagellum again propagated waves from tip to base.

When the micromanipulator probe was used to constrict a central portion of the flagellum, the proximal region behaved in a similar way to that described above for the whole flagellum constricted at the tip. If a central or basal portion of the flagellum was constricted the distal region could propagate waves both from and towards the obstruction and occasionally executed local oscillatory movements. Reversal of the direction of wave propagation and the onset of local oscillatory motion appeared to occur randomly and could not be induced by increasing the pressure exerted by the probe on the flagellum. There was no correlation between the external behaviour of the portions of the flagellum on either side of the obstruction.

Severing the flagellum at a central region resulted in waves propagated from the base on that portion still attached ot the cell. Occasionally, after some time had elapsed, waves were observed to be propagated from the severed region towards the base. The behaviour of the severed distal fragment depended both on the speed with which the flagellum was severed and on the direction of wave propagation at the time of cutting. If the bend propagation was from tip to base and the flagellum was severed sufficiently rapidly that wave reversal did not occur, then the waves on the free-swimming segment were initiated at the tip. The severed fragment of a flagellum initially propagating waves from base to tip continued to propagate waves in this direction, initiating them at the point of amputation. Severed portions were not observed to reverse their directions of wave propagation. The length of time for which a free-swimming fragment beat in culture medium or Ringer's solution was very short, at most a few seconds. When the amputation was performed in a medium containing ATP, the flagellar fraction beat for between 30 and 60 sec. The effects of constricting or dissecting the flagellum are summarized in Table 1.

Contact between the probe and the body of a free-swimming organism which propagated waves from tip to base along its flagellum produced one of two effects. Either the direction of wave propagation was reversed or the frequency increased markedly. In some organisms with stationary flagella, touching the body with the probe induced flagellar activity.

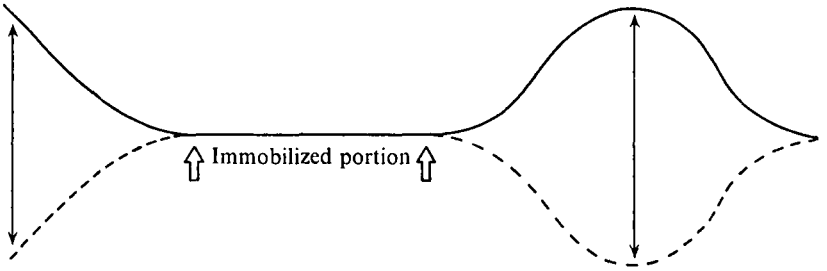

Fig. 1. Behaviour of flagellum immobilized between the two broad arrows. The other arrows indicate the direction of movement of the free flagellar regions.

By careful micromanipulation the membrane covering the cell body could be broken, thus allowing the contents of the cell to escape and exposing the basal structures of the flagellum to the surrounding medium. In Ringer's solution or in culture medium flagellar motility ceased after a few seconds, but if the micro-dissection took place in a solution containing ATP and magnesium ions (see material and methods), motility was maintained for an average of 10 min, with a maximum of 30 min. The direction of wave propagation following this procedure was always towards the tip, irrespective of the initial direction of propagation. The amplitude of propagated waves increased and the waveform became meander-like (see e.g. Silvester & Holwill, 1972). Towards the end of their active period the flagella adopted a more extended wave shape and the frequency fell to about 5 Hz; the addition of fresh ATP to the solution at this time caused the frequency to increase. It was not found possible to reverse the direction of wave propagation by touching any region of the isolated flagellum with the micro-needle. Constriction of any point along the flagellum frequency caused complete cessation of movement. On those flagella where movement was observed constricting the basal region had little effect on the movement, whereas a constriction in the central region of the flagellum induced large-amplitude, low-frequency waves on the basal side of the needle and had little effect on the wave motion on the distal side.

In some organisms a considerable portion of the flagellum became attached to the coverslip and left a central and distal region moving, as shown in Fig. 1. In these cases the movements of the two regions were always of the same frequency and maintained a constant phase relationship relative to each other, i.e. the movements were highly correlated.

DISCUSSION

Kaneda (1965) has described the results of experiments in which a microplate was used to mechanically obstruct waves on frog sperm tails. As for the flagellum of *Crithidia*, the portions of the sperm tail proximal and distal to the probe were able to beat, and since they could beat at different frequencies there was no correlation between the beating of the two parts of the flagellum. For sea-urchin spermatozoa Gray (1955) has reported that waves are unable to pass an obstruction, so that although the proximal region may be active the portion distal to the obstruction is motionless. The responses of the two sperm tails are different, both one from the other and from the flagellum of *Crithidia*. It is important to note, however, that the effects of the force exerted by a microprobe on the sperm tails have not been studied and may be respon-

sible for the apparent difference in behaviour between the two. Thus, increasing the pressure of the microprobe on the frog sperm tail may prevent movement of the distal region as observed for the sea-urchin spermatozoon. Conversely, a less restrictive obstruction might allow beating of the distal portion of the sea-urchin sperm tail. Critical studies, using the methods described in this paper, on flagella which propagate waves in one direction only, such as the sperm tails discussed above, would provide further information of relevance to the specification of the bending mechanism within flagella.

The work described in this paper is related to the laser irradiation studies applied to *Crithidia oncopelti* and described by Goldstein *et al.* (1970). In the laser work the flagellum suffered localized damage when a pulsed ruby laser microbeam was focused on it. In most cases the effects of microdissection with the laser are similar to those with the microprobe and confirm that any portion of the flagellum of *Crithidia* can initiate waves and that an isolated flagellar fragment can continue to initiate and propagate waves. In respect of wave initiation, as has been remarked elsewhere (Goldstein *et al.* 1970), the flagella of *Crithidia* differs from those of other organisms, which appear to require their basal regions to initiate waves (Goldstein 1969, Kaneda, 1965), although the region of initiation need not be near the base (Brokaw & Gibbons, 1973).

One important difference in flagellar response to the two treatments discussed above is associated with behaviour following damage to the tip of the flagellum. If such damage is effected by laser radiation, the ensuing wave propagation is invariably from base to tip; on no occasion was a proximally directed wave observed. After dissection of the flagellar tip, using the micromanipulation procedures described in the present study, wave propagation was frequently observed to be from tip to base. The major difference between the effects of the two procedures probably lies in the physical characteristics of the tip following their application. After laser irradiation the components within the flagellar tip are probably welded together as a result of intense local heating. With mechanical dissection using a probe, on the other hand, the components are expected to be relatively free from each other. In particular, the fibrils in an irradiated flagellum might not be free to slide at the tip, whereas in the mechanically dissected flagellum sliding is likely to be unrestricted. These arguments suggest that free filament sliding is necessary for the initiation of waves from the flagellar tip, although an alternative possibility, that mechanical manipulation damages specialized initiation structures less than irradiation procedures, cannot be eliminated on the basis of evidence presently available. It is interesting to note, however, that the tip structures of *Crithidia* are essentially the same as those in organisms which propagate waves in one direction only (Burnasheva, Ostrovskaya & Yurzina, 1968; S. F. Goldstein, personal communication).

A further difference, but one which is closely associated with that just described, between the irradiated flagella and those severed by micromanipulation lies in the direction of propagation of waves on that portion of the flagellum which remains attached to the cell. On manipulated flagella waves were observed to be propagated from the damaged end towards the base, whereas waves were never observed to be propagated in this direction on irradiated flagella. This supports the suggestion that free movement of flagellar components is required for wave initiation. Neither of these

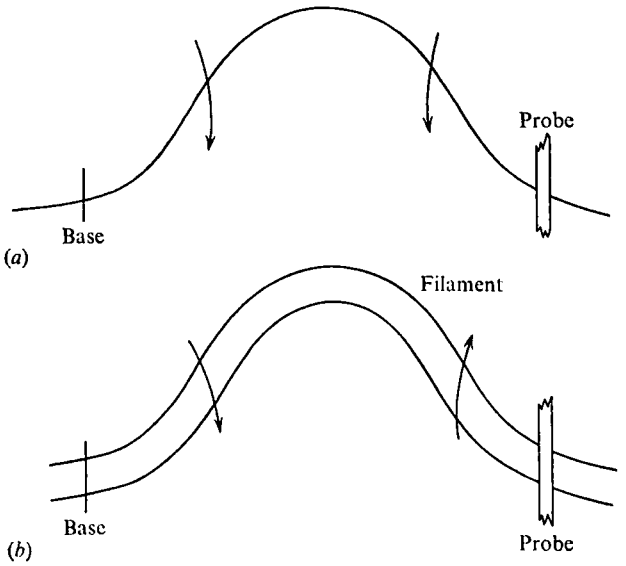

Fig. 2(*a*). Directions (arrowed) of moments necessary to produce local oscillation of a flagellum between the two fixed points. (*b*) Directions (arrowed) of moments generated by a sliding filament system if normal wave propagation is attempted.

observations on manipulated flagella supports the conclusion of Goldstein *et al.* (1970) that the basal region acts as the dominant wave initiator when the flagellar tip is removed. A critical comparative electron-microscopic examination of the damage caused by micromanipulation and laser irradiation might provide more information about the mechanism of wave initiation.

The different behaviour of irradiated and manipulated flagella has been interpreted in terms of the relative freedom of movement of components within the flagellum, and is better related to a sliding filament system than to a local contraction system, in which restriction of relative movement would not be expected to modify the behaviour significantly. Further observations which support a sliding-filament system arise when the flagellum is constricted in a central region or at its tip by means of a microprobe. The reduction of frequency which occurs as the pressure of the probe on the flagellum is increased can easily be understood if the result of the pressure is to impede relative sliding of filaments within the flagellum.

The local oscillation which occurs as the pressure exerted by the probe on the flagellum is further increased can also be explained using a sliding filament model, but the distribution of forces along the flagellum must be different from that arising in the normally beating organelle. To achieve local oscillation, the bending moments must be as shown in Fig. 2(*a*). In a sliding filament model comprising two filaments, relative sliding is prevented by basal structures and by the action of the probe. Consideration of the system shows that, if the flagellum is attempting to propagate waves from base to tip, the bending moments at base and probe areas are as shown in Fig. 2(*b*), so that local oscillation will not occur. If, however, efforts are made by the flagellum to propagate waves both from the base and from the obstructed region the condition of Fig. 2(*a*) could develop and local oscillation could occur. If flagellar bending is pro-

duced by a local contraction mechanism, events which occur at a particular point within the flagellum do not influence remote regions of the flagellum by internal mechanical communication, as is possible in the sliding filament model. The presence of the probe therefore might not be expected to alter significantly the pattern of bending between the flagellar base and the probe. However, if the pressure exerted by the probe on the flagellum provides a stimulus whereby forces arise to propagate waves from the probe, as well as from the base, the moments shown in Fig. 2(*a*) might arise and local oscillation could then develop.

A further observation which favours the sliding filament system is the correlation observed between movements of a distal and central region of the flagellum when the intervening portion is immobile. The correlation could easily be achieved by filaments sliding within the immobilized section, but communication between the two regions in a local contraction model would require more complex information transfer.

The requirement that, after the removal of a constriction, the wave present on the flagellum must be propagated to the tip before initiation from that region can occur may reflect a property of the wave-propagation mechanism associated with direction. Thus, it may be that a wave initiated at the basal end (say) is automatically propagated to the tip by virtue of the properties of the system. This is not unreasonable on a flagellum capable of wave propagation in two directions, since it precludes the possibility of waves being propagated simultaneously in both directions from a mid-region of the organelle. More detailed analyses using high-speed photography of the conditions associated with wave initiation may provide further information about this important feature.

The tactile response of the cell suggests that flagellar reversal is preceded by a signal carried by the cell membrane. The signal appears to be a transient one, since in the event of maintained stimulation the flagellum reverts to the propagation of waves from tip to base, and further stimulation of other regions of the cell frequently fails to induce reversal again.

The unidirectional character of flagellar waves following dissection of the cell body suggests that the body has a controlling influence on the wave direction. This may be associated with the mechanical coupling between the flagellum and cell or with the intracellular chemical environment. This type of preparation provides an additional experimental system on which to study the behaviour of flagellar waves in response to changing chemical environments. The system is clearly different from that of glycerol-extracted or detergent-extracted flagella since in the latter the membrane is modified or dissolved completely (e.g. Gibbons & Gibbons, 1972). The characteristics of the new preparation are at present being studied in our laboratory.

In this paper we have shown that the techniques of micromanipulation can be used to study the responses of flagella to mechanical stimulation. The results are generally consistent with a model containing inextensible sliding filaments, although no conconclusion can be reached about the active or passive nature of the sliding.

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SUMMARY

1. The behaviour of the flagellum of *Crithidia oncopelti* when subjected to constriction and dissection by a microprobe was investigated.

2. Constriction of the flagellar tip resulted in wave reversal, whereas dissection of this region allowed normal wave movement.

3. Constriction of the basal region allowed both base-to-tip and tip-to-base propagation.

4. The observed behaviour is more easily explained in terms of a sliding filament model of flagellar movement than by a model which involves local contraction.

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