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Original article

Dinoflagellate flagella adopt various conformations in response to different needs

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Summary – The two flagella of Dinoflagellates have, up to now, been poorly described. They display different structures and different patterns of behaviour compared with other organisms. In addition, the two flagella are different from each other: the transverse flagellum is ribbon-shaped and beats with a spiral undulation inside a furrow located around the cell body while the longitudinal flagellum has a larger diameter than simple flagella because it contains structures in addition to the axoneme and propagates essentially sinusoidal waves to push the cell. *Ceratium* flagella are particularly interesting to study because they both show different types of movements and have complex structures in addition to the axoneme. We propose that the additional structures are responsible for the particular movements of Dinoflagellate flagella. The presence of food particles in vacuoles in the vicinity of the flagella pocket suggests that their flagellar apparatus may not only be a propulsive organelle but could also be involved in prey capture.

protist / flagellar movement / flagellar contraction / nanofilaments

Introduction

The behaviour, ultrastructural and molecular structure and function of cilia and flagella have been well documented over the past 20 years [17, 29]. These microtubule-based organelles are responsible for the motility of organisms and usually beat with planar, nearly sinusoidal, waves [4] as in the case of sea urchin spermatozoa (Brokaw, 1965). The structure and behaviour of the flagella of the Dinoflagellates have not previously been described in detail. Dinoflagellates are bi-flagellated but their two flagella show different structures and beat differently [19]. The transverse flagellum is ribbon-shaped, with the appearance of a cork-screw wrapped around the cell inside a furrow, the girdle, and propagates 3-D waves [16, 28]. The longitudinal flagellum is oriented backwards and supports almost sinusoidal waves. Both flagella are involved in the motility of the organism: the longitudinal flagellum induces forward movement of the cell whereas the transverse one causes its rotation. Apart from this general pattern, the longitudinal flagellum of some species (*Ceratium*) is also able to retract like a coiled spring [18, 20, 26]. In the present paper, we attempt to understand this behaviour and propose explanations of these unusual flagellar movements.

Materials and methods

Biological materials

The marine organisms were collected from superficial layers (0 to 10 m depth) with a thin meshed (50 μm) net in the bay of Villefranche-sur-mer during the autumn and spring months. Individual cells were isolated with small pipettes (*ca* 50 μm in diameter) and maintained in Petri dishes containing sea water until video-recordings or fixation for electron-microscopy were made. *Oxyrrhis marina* was cultured as described in [13].

Several species of *Ceratium* were studied, *C furca* Ehrenberg, *C tripos* Müller, *C fusus* Ehrenberg, *C candelabrum* Ehrenberg, *C gravidum* Gourret and *C limulus* Gourret. These "armoured" species possess a theca made of thick cellulose plates and bear long spines, the apical one being longer and open at its extremity. The cells are almost flat, the dorsal side being convex while the ventral one is concave. Their two flagella originate from a shallow cylindrical pocket, the flagellar pocket, in the middle of the ventral area.

Motility observations

These were made using Leitz and Reichert microscopes equipped with differential interference contrast (DIC) Nomarski optics. Video recording were made with a Panasonic CCD camera (F 10) combined with a Hamamatsu real-time image processor (DVS-3000) using a Sony U-matic or a Panasonic Super VHS video tape recorder. Some sequences were obtained using stroboscopic lighting at 50 Hz frequency. Photographs of the screen were obtained on Technical Pan 2415 Kodak film.

Electron microscopy

Fixation was carried out according to that described in [21]. The cells were fixed with a 0.1 M phosphate buffered fixative containing 5% glutaraldehyde, 0.8–1 M glucose pH 7.4–7.8 at room temperature for 1 h. They were washed in buffer with 0.3 M phosphate and 0.8 M glucose. After treatment by 2% OsO_4 in phosphate-glucose buffer for *ca* 1 h, a decreasing graded series of phosphate-glucose solutions were used, before a progressive dehydratation. The cells were embedded in Spurr's low viscosity medium [27]. The sections were stained with 9% uranyl acetate in methanol followed by lead citrate and examined with a Hitachi H603 electron microscope.

Because the fixative acts as a contracting agent and calcium as a triggering retraction agent for nanofilament structures,

Ca^{2+}-free artificial sea water was used to prevent contraction of flagella (477 mM NaCl, 97 mM KCl, 20.9 mM $MgCl_2$, 27.6 mM $MgSO_4$, 5 mM ethyleneglycol bis (β aminoethylether) N,N-tetraacetic acid (EGTA) and 30 mM Tris HCl (pH 7.6). The living cells were briefly washed in this medium prior to fixation.

As flagella are preserved *in situ* with difficulty, the cells were trapped among fibers of nucleohistones (Hubert *et al*, 1962, *J Micr* 1, 163–165) for ease of manipulation. The flagella often break when *Ceratium* are manipulated, but are regenerated within 2–3 h.

Results

We compared the behaviour and structure of *Ceratium* flagella to that of *Oxyrrhis marina* flagella whose structure and function has been extensively studied [10, 12] and which will serve as a reference model throughout this paper.

The transverse flagellum

Behaviour

The transverse flagellum originates from the bottom of a "flagellar pocket", which is generally cylindrical and located on the ventral side of the cell. It is ribbon-shaped and generates complicated waves along a curved corkscrew axis (fig 1).

Analysis of video-recordings of the swimming of *O marina* showed that the transverse flagellum beats from the base of the tip, propagating helical waves around the cell body and causing the organism to rotate in the opposite direction. When it is detached from the cell body, permeabilized and reactived with ATP, it also propagates helical waves which cause a 3-D movement. In both situations, attached or detached, it beats in the same manner. This suggests that the beat is an intrinsic property of the transverse flagellum. In *Ceratium* the persistence of the coiled sinusoidal waves is observed when the flagellum is extruded from the girdle, for instance after transfer in Ca^{2+}-free sea water (fig 2).

Structure

Parallel to the axoneme runs a bundle, the "striated strand" made of 2–4 nm filaments (nanofilaments [12]) surrounded by cytoplasm (fig 3). In the flagellum this strand is opposite the axoneme and is close to the cell-body membrane at the bottom of the girdle. In *Oxyrrhis marina,* the striated strand is about 6 nm in diameter and is periodically linked every 16 nm to an outer doublet of the axoneme. Granular dense spheres about 20 nm in diameter are observed every 40 nm along this bundle. The nanofilaments are tightly coiled, and have the appearence of dense spheres. In *Ceratium furca*, a much larger species, this bundle reaches up to 250 nm in diameter. It appears striated in Ca^{2+}-free sea water, the striae corresponding to coils of the filaments.

The basal body of the transverse flagellum is generally oriented roughly perpendicular to that of the longitudinal flagellum. The proximal portion of the transverse flagellum bears thin hairs, the mastigonemes, which seem to adhere to the bottom of the girdle.

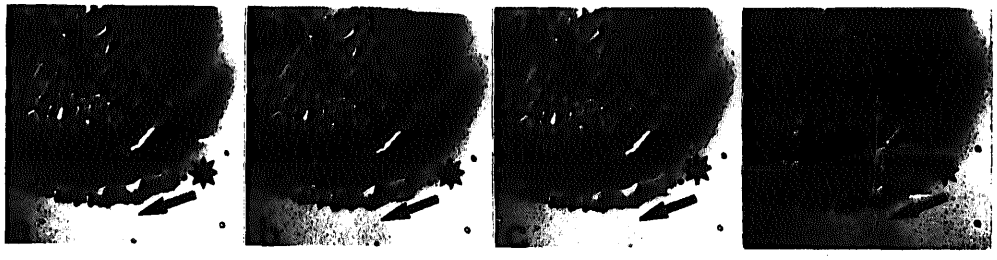

Fig 1. Sequence from a video-recording of the beating transverse flagellum of *Ceratium tripos*: its spiral waves propagates inside the girdle. *emergence site of the flagellum. ← direction of wave propagation. Numbers refer to time in milliseconds (× 800).

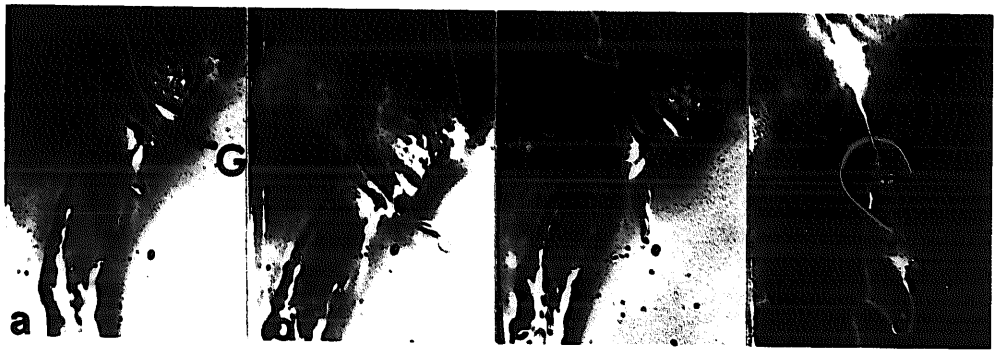

Fig 2. The distal part of the transverse flagellum (arrow) of *Ceratium gravidum* beats outside the girdle, in Ca^{2+}-free sea water (× 800). (Time interval between video frames is several seconds). The helicoidal waves gradually stop (**a, b**) and the transverse flagellum relaxes outside the girdle. (**d**) = ← G.

The longitudinal flagellum

Behaviour

The longitudinal flagellum shows three distinct types of movements in *Ceratium* whereas it shows only two in *Oxyrrhis:*

– Originating from the bottom of a large (*ca* 4 μm) flagellar pocket, it propagates almost sinusoidal waves which are not planar, but clearly tridimentional. They can be observed more easily when the cells are stuck on a glass-slide. In *O marina,* the wave amplitude is larger at the proximal part of the flagellum and decreases regularly towards the tip. Its beating initiates at the very base of the flagellum. Waves are propagated inside the flagellar pocket in *Ceratium* with low amplitude. After the narrow aperture, a node of the oscillation, it reaches its maximal amplitude (fig 4).

Once isolated, the longitudinal flagellum of *Oxyrrhis* beats symmetrically. When it is detached from the cell body (for instance by a sudden Ca^{2+} rise), it keeps the same linear trajectory for a few s. The beating amplitude becomes much lower than before, but the frequency remains the same;

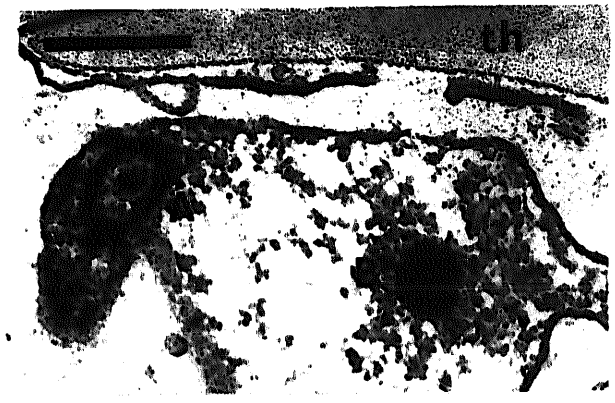

Fig 3. Electron micrograph showing a transverse section of the transverse flagellum of *Ceratium furca* inside the girdle. It shows the bundle of nanofilaments and the axoneme. The nanofilaments coil forming tubes of *ca* 10 nm in diameter. th: theca of the organism; A: axoneme; B: bundle of nanofilaments. × 80000; bar = 0.25 μm.

– *In vivo* the longitudinal flagellum is able to fold suddenly and spontaneously or in response to several triggering agents such as a mechanical shock or light change, inducing a change of the swimming direction of the organism. This is known as the avoidance reaction.

In *O marina* the flagellum switches from a backward orientation to a nearly forward position, folding rapidly (1/20 s) towards the cell body and then resumes its normal backward position [12]. This behaviour can also be triggered when the cell meets an obstacle or is submitted to light change.

This avoiding reaction becomes extremely frequent whenever *Oxyrrhis* is close to, but not necessarily in direct contact with a particle or near other *Oxyrrhis* compatible cells. It swims in close circles for several s before ingestion of the particle or fusion with the cell. This behaviour might be triggered by some kind of chemotaxis. The frequency of the avoiding reaction is also increased when Ca^{2+} ions reach a concentration higher than in normal sea water, *ie* more than 10 mM.

In *Ceratium*, the observations of free organisms clearly show that the "avoiding reaction" is not necessarily triggered by the presence of a particle. This reaction consists of a forward bending of the longitudinal flagellum, while the axoneme keeps beating, therefore inducing a backward swimming. During the reorientation, the flagellum continues to beat on one side of the cell body, in various intermediate positions between the transient anterior one and the normal posterior one. This is responsible for the direction change. In *Oxyrrhis,* a fast bending, which stops the axonemal beating, is used to reorient the swimming direction.

– In *Ceratium*, apart from the behaviour described above, the longitudinal flagellum is also able to retract suddenly (fig 5). The retraction is fast (less than two video frames, or 1/50 s). First wave propagation ceases along the flagellar length, followed immediately by the retraction process. The whole length of the flagellum coils very tightly into at least 10–12 regular folds (fig 5d) before completely disappearing inside the flagellar pocket (fig 5e, f). The folds are initially loose, and tighten progressively with time. They are planar outside the pocket but appear to coil into a non-planar configuration when they are finally inside the cylindrical flagellar pocket.

A few seconds (2 to 10 s) later, the flagellum slowly reextends, first inside and then outside the pocket (fig 5g to r) and progressively (*ca* 1/2 s) undulates as previously (fig 5s, t). The proximal portion is first unfolded while the distal tip remains coiled for a longer period (fig 5 j, k, l) until the original sinusoidal pattern is recovered (1/10 s).

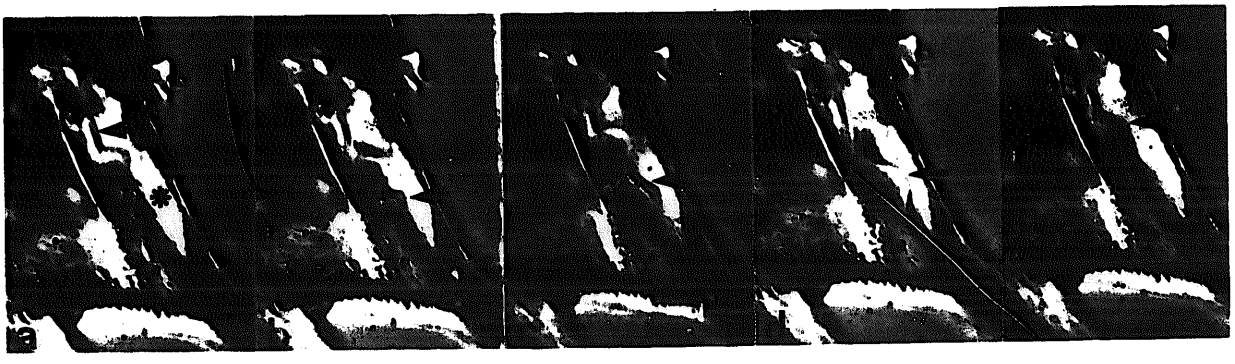

Fig 4. Video sequence of the beating longitudinal flagellum (arrow) of *Ceratium furca* inside the flagellar pocket (*). Numbers refer to time in milliseconds.

This retraction appears after a mechanical stimulation, as suggested in [20] or from time to time without any obvious stimulus. It can be induced by a free Ca^{2+} concentration rise on a permeabilized model *in vitro* [24].

Similarly, we should also mention the resting position in *Oxyrrhis* during which the longitudinal flagellum is completely and reversibly folded around the hyposome at the back of the cell body.

Structure

The diameter of the longitudinal flagellum is much larger than that of a flagellum such as the sea urchin spermatozoa containing an axoneme alone. The flagellum is wider only in the first three-quarter proximal part, which is clearly visible by DIC or dark field microscopy. This is due to the presence of the associated-structure, the paraflagellar rod (PFR), that runs along the proximal

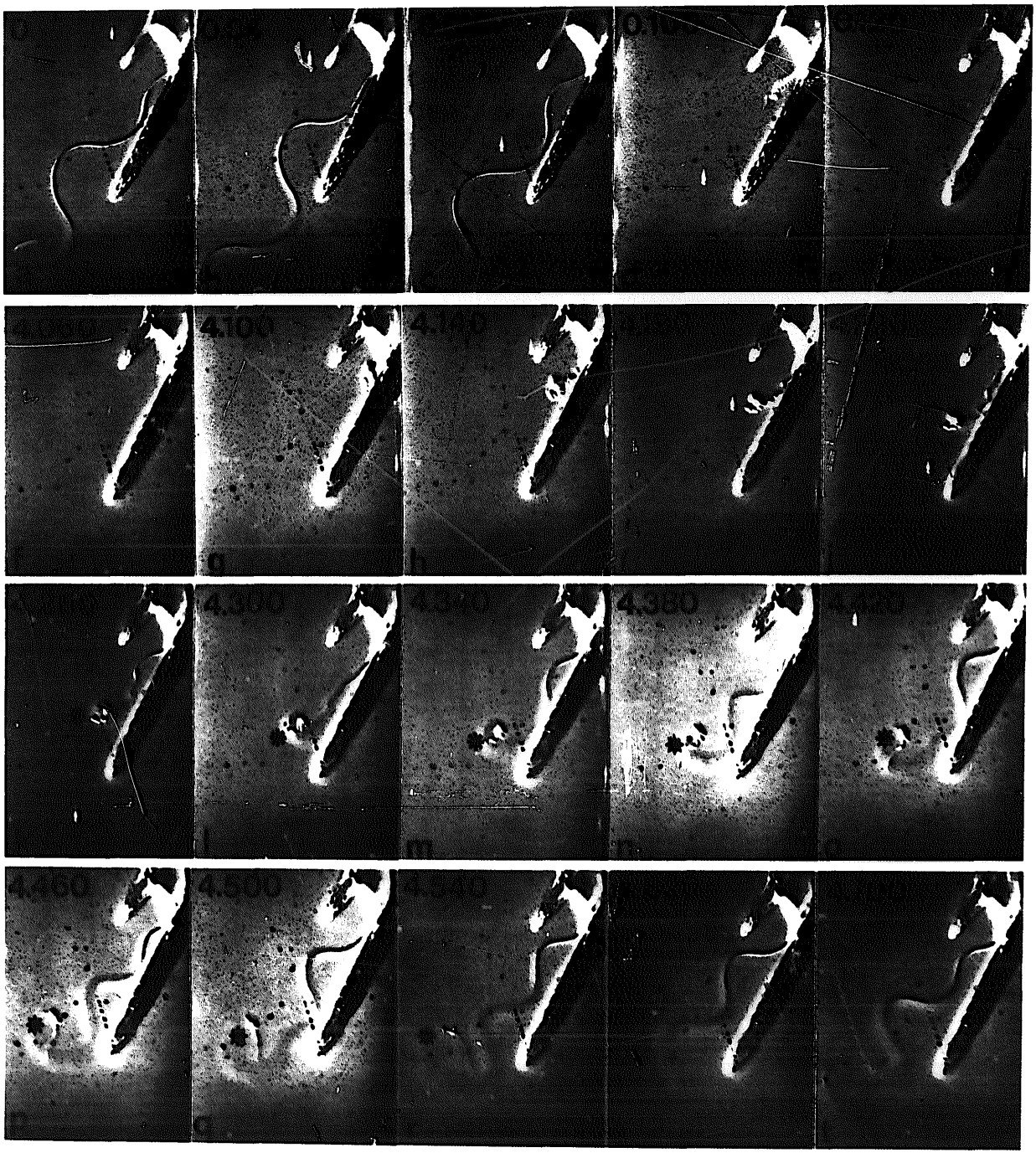

Fig 5. Sequence from a video-recording of the longitudinal flagellum of *Ceratium furca* which propagates helical waves (**a, b, c**), which retracts (**d, e**) and disappears inside the flagellar pocket. It re-extends (**f–r**) progressively before beating again normally (**s-t**). In *ca* 1 s, its proximal part stretches progressively while its distal extremity remains "crumpled" for a while (★). Numbers refer to time in seconds (× 800).

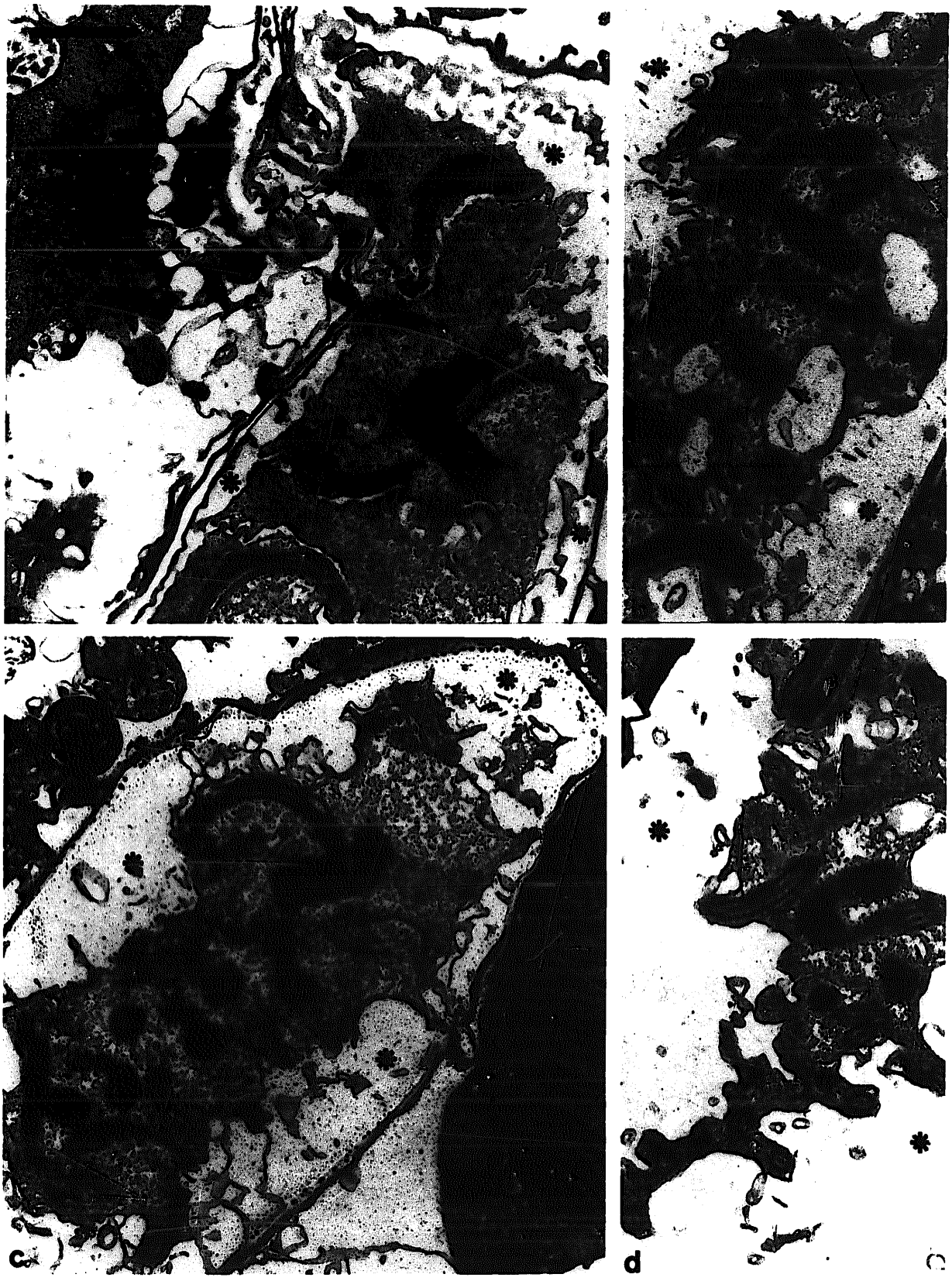

Fig 6. Electron microscopical sections of the flagellar pocket of *Ceratium furca* (★) including a retracted longitudinal flagellum (× 20000; bar = 1 μm). **a.** Tangential section: the axoneme makes large arches around the R-fibre (Rf) which is along the axis of the flagellum when retracted. **b.** This section is more deeply located. The axoneme sections are always near the flagellar membrane (arrow) and the paraflagellar rod always inward. Many invaginations of the membrane are observed. **c.** In this section the R-fibre is linked to the PFR (>) and its axial part is dense. **d.** Section of the distal extremity of the flagellum. Neither the paraflagellar rod, nor the R-fibre are seen along the tip. This section explains the appearance of "crumpled paper" seen in video frames of figure 5 (h to q) when the flagellum re-extends.

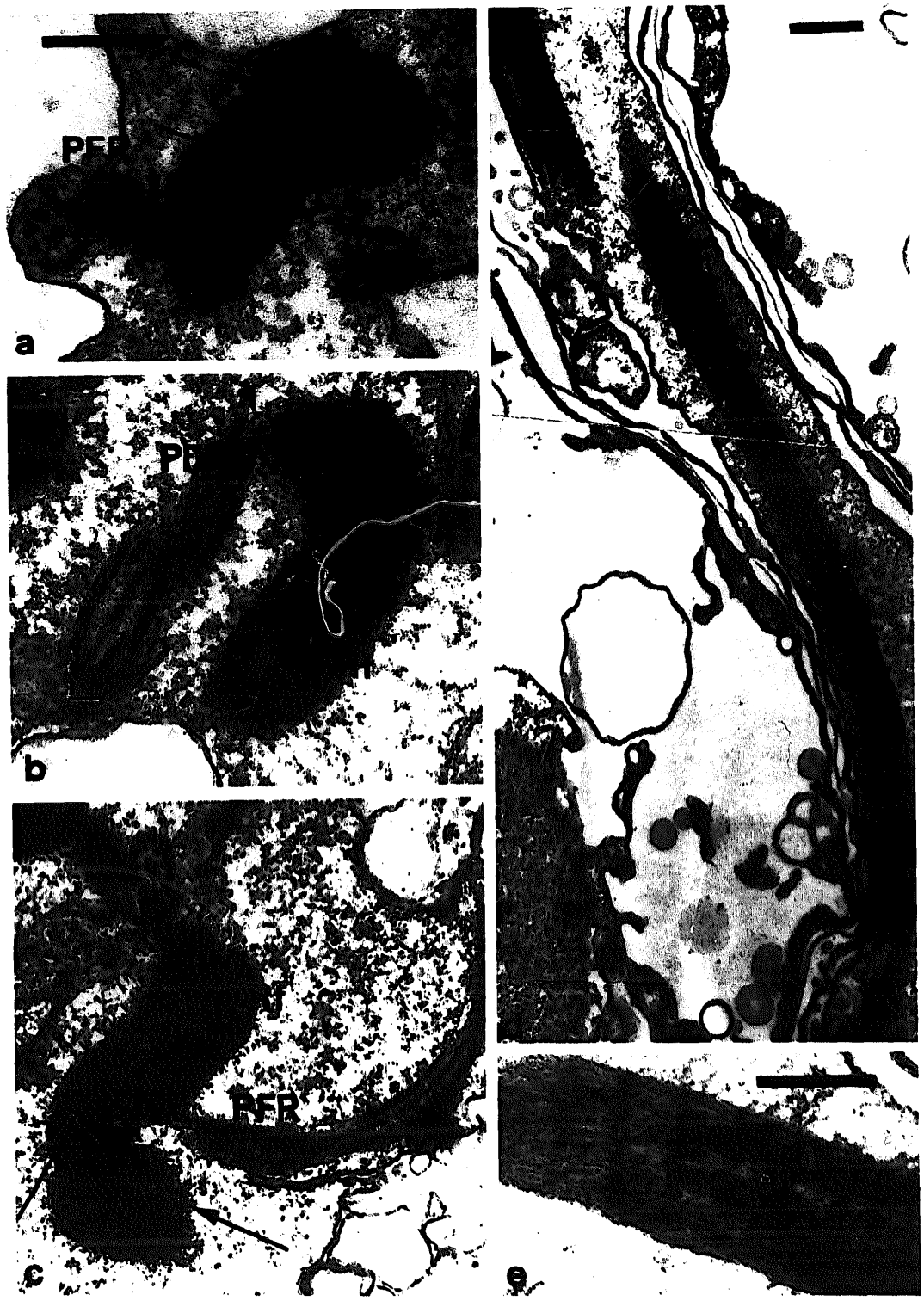

Fig 7. a, b, c. Electron micrographs of *Ceratium furca* showing details of the various connections (short arrows) between the paraflagellar rod (PFR) and the R-fibre (Rf). The coiled nanofilaments can easily be observed (long arrows); they appear continuous with those of the PFR. × 60000; bar = 0.5 μm. **d.** The longitudinal flagellum is sectioned longitudinally. Due to fixation conditions (*ie* low Ca^{2+}) it appears relaxed so that the periodicity of the bundle of nanofilaments is easily observed. × 25000; bar = 0.5 μm. **e.** The nanofilaments of the R-fibre are, in this section, parallel and have a periodic structure. × 80000; bar = 0.25 μm.

3/4 of the axoneme. This structure has been seen in all longitudinal flagella of Dinoflagellates that we have examined [10].

As in *Oxyrrhis,* the PFR (fig 6a, b, c) of *Ceratium* is made of a cylindrical structure composed of two elements generally close to doublet no 5 and 6, an outer and an inner one similar to that of *Euglena* [11]. The outer-PFR is up to 70 nm in width, crescent-shaped, and contains 8 layers of filaments, whereas the inner-PFR, which also contains 8 layers of filaments, is straighter and thinner (20 nm) [12]. The filaments of the outer-PFR are perpendicular to those of the inner-PFR which gives these structures an appearance of a pantograph (fig 7b, c). The pantograph model assumes that bundles of filaments are linked at their crossing points: the contraction of individual filaments could in this way amplify the shortening of the whole PFR structure while saving its stiffness and compaction.

In *Oxyrrhis,* this paraflagellar rod is the only structure associated with the axoneme in the longitudinal flagellum. In larger species, other additional filamentous bundles are observed, one of them in *Ceratium tripos* [21] being very large and reaching 0.5 μm in diameter (the R-Fibre). We observed it also in the different species of *Ceratium* we have studied (figs 6, 7). The R-fibre becomes thinner and disappears before reaching the distal extremity of the flagellum (fig 6d). The axial core is denser than the peripheral material and may be related to the contraction state. Another fibre, the striated fibre, is much thinner (50 nm in diameter) and is close to doublet no 7 in most sections.

All these associated structures are made of thin filaments of 2–4 nm diameter. In fixed organisms, the filaments always appear to be coiled (*ca* 10–12 nm in diameter), the fixative acting as a triggering agent for contraction (fig 7a, c). In contrast, when the cells are fixed in a Ca^{2+}-free sea water fixative, the filaments seem to be relaxed, and have a periodic structure (fig 7d, e) as observed by Maruyama [21].

Inside the flagellar pocket, the membrane of the retracted flagellum which is tightly coiled (fig 6b, c), invaginates into the cytoplasm and makes tortuous paths joined side by side exactly like desmosomes. In transverse sections, the axonemes are always located close to the cell membrane as if they were linked to it, the paraflagellar rod being located on the inner side of the axoneme (fig 6b). The large bundle of nanofilaments of the R-fibre is observed parallel to the main axis of the flagellar pocket (fig 6a, b, c) in most cases.

When the flagellum is contracted, the axoneme with its paraflagellar rod makes semi-circular arches (fig 6a) which, according to ultrastructural analysis, are regularly linked to the nanofilaments of the R-fibre and physically interconnected to the paraflagellar rod (fig 6c, 7a, b, c).

The cytostome and the excretory apparatus (pusule) are in the vicinity of the two basal bodies, as is the case in other dinoflagellates [6]. Many small food particles are always observed inside the flagellar pocket between the flagellar arches. In addition, sections of *Ceratium* behind the cytostome show large food vacuoles including particles and large preys (Cachon *et al,* unpublished results).

Functional significance of the flagellar beatings

Flagella and cilia are involved in the motility of the organisms by exerting a propulsive force on the surrounding medium. They swim while rotating, since their two flagella beat according to two distinct patterns: the transversal one is partly responsible for the rotation, while the longitudinal one pushes forward. In addition, it follows an helicoidal path. So far we cannot explain this helicoidal path simply from the beat pattern of two flagella.

Why does an organism move in space? Mostly because it looks for better conditions, including food. As an adaptation to planktonic life [9], several possibilities allow a *Ceratium* cell to find food:

Forward swimming

This is mostly due to the basic pattern of flagella beating that pushes the cell body as in spermatozoa, dinoflagellates, kinetoplastids and alga (*Chlamydomonas* for example). In dinoflagellates, the longitudinal flagellum assumes this function. However, *O marina* when devoid of longitudinal flagellum displays progressive forward swimming which is much slower and erratic ([16]; personal observations).

Rotation swimming

The transverse flagellum is mostly responsible for this type of swimming. It undulates around the cell body inside the girdle and makes the cell turn around its main axis. As a consequence, it moves particles in circumferential waterstreams (fig 1) in "the reverse sense of beating" as already described by Gaines and Taylor (1985). These streamings will eventually carry food particles towards the cytostome which is located in the vicinity of the flagellar base, especially when the cells are resting, which is typical enough of dinoflagellates to motivate their group name (dinos = streams in greak).

Avoidance behaviour

To avoid obstacles in its way, or high light levels, the longitudinal flagellum of *Ceratium* bends towards the anterior part of the cell, ("avoiding reaction"), which is insufficient to tilt the cell body which presents a too high drag due to its large size as compared to *O marina* where this tilt is observed [13]. As the axoneme continues to propagate waves, the folding induces a backward swimming. This is followed by a slow unfolding (a few seconds) during which the flagellum beats in a variable direction which induces the rotation of the cell body until the flagellum reaches its original position, trailing behind the cell body.

Retraction of the longitudinal flagellum

Ceratium is an organism which possesses a thick theca. It would be unable to feed without a structure such as its longitudinal flagellum which moves food particles towards its cell body and especially towards feeding organelles where particles are ingested. This flagellum acts as does the peduncle of *Erythropsidinium* or of *Noctiluca* [9]. Its folding induces the formation of streams carrying food particles towards the cytostome through which particles are ingested. In *Ceratium furca* the presence of food particles into vacuoles was observed in the vicinity of the flagellar pocket: this suggests that the retraction of the longitudinal flagellum would be implied in the process of prey capture. Though *Ceratium* is photosynthetic because it possesses plasts, a phagotrophic feeding process has been observed (*C lunula* [23]).

The behaviour of these flagella could represent an adaptation to food collection during the planktonic life for these Dinoflagellates [9].

Discussion

The axonemal associated structures, such as the paraflagellar rod, the striated fibre, the R-fibre, are involved in different types of behaviour of the flagella of dinoflagellates. The unusual pattern of contraction of *Ceratium* flagella suggested by Maruyama [20] that structures additional to the axoneme could be responsible for the contraction mechanism [21]. Using permeabilized models, Maruyama [22] established that the flagellar contraction could be controlled by adjusting the Ca^{2+} concentration in the absence of ATP.

The waves of the longitudinal flagellum are due to the ATP-dependant sliding of the axonemal microtubule doublets as in all cilia and flagella, and we suggest that the coiling of the transverse flagellum is induced by the Ca^{2+}-dependant contraction of the smaller striated fibre. The avoiding reaction associated with the behaviour of the longitudinal flagellum seems to be triggered by the paraflagellar rod contraction (this suggestion was first made for the flagellum of *Oxyrrhis*, in which there is no other associated structure) and the retraction of the longitudinal flagellum seems to be induced by the contraction of the R-fibre. This fibre is able to contract highly because when retracted it is much shorter, but reaches a larger diameter with a denser axial core.

The R-fibre, the PFR and the striated fibre are all made of nanofilaments about 2-4 nm in diameter which are contractile, but do not contain actin. The presence of transverse striations showing a variable periodicity is probably due to the state of contraction or relaxation of the filaments, the dark bands corresponding to locally coiled segments as in flagellar rootlets [3, 6, 24]. These structures should simply be termed "myonemes" as they appear very similar to those observed inside many cells. They are responsible for the contraction of organelles or organisms [1, 6, 7, 10, 14, 15].

These 2-4 nm nanofilaments, which are able to contract by coiling in the presence of calcium ions without any direct requirement for ATP [22, 23] are present in all kinds of eukaryotic cells, including mammalian ones [25]. Moreover, intermediate filaments (10 nm in diameter) of higher eukaryotic cells are made of 2–3 nm protofilaments [2]. Like the latter, nanofilaments constitute a distinct class of filaments of the cytoskeleton, identical in size and organisation, even though they are made of various kinds of proteins and are present in different cellular locations involved in different functions. One can distinguish different types of nanofilaments by means of immunological techniques (unpublished observations). Their study could bring new information about their function as well as their structural and functional relationship to intermediate filaments.

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