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SPERM CHEMOTAXIS IN SIPHONOPHORES

II. CALCIUM-DEPENDENT ASYMMETRICAL MOVEMENT OF SPERMATOZOA INDUCED BY THE ATTRACTANT

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SUMMARY

Spermatozoa from siphonophores have been shown to be attracted towards an extracellular structure, the cupule, which covers the predetermined site of fertilization of the egg.

Observations on sperm behaviour during the chemotactic response show that spermatozoa describe trajectories of large diameter (700–1000 μm) while far from the cupule, and of smaller diameter (200 μm) in the cupule area. The transition between the two types of swimming occurs progressively when spermatozoa cross a 3 mm wide area around the cupule. After a few minutes 99 % of the spermatozoa keep swimming around the attractant source, following circular paths 150–200 μm in diameter.

In the absence of the attractant, comparable modifications of sperm trajectories are observed in the presence of the ionophore A23187 and high calcium concentrations. In the presence of 10^{-2} M calcium ions, A23187-treated spermatozoa describe trajectories 200 μm in diameter, which increase up to 800 μm at lower calcium concentrations (10^{-6} M).

In the absence of calcium ions, spermatozoa swim across the cupule area without modification of their trajectories and no sperm accumulation can be detected. This requirement of the chemotactic response for calcium ions is observed either with fresh cupules stuck on the eggs, with cupules separated from the eggs, or with cupule extracts. Moreover, a soluble component fractionated from the cupule induces, when diluted in sea water, a reduction in the size of the sperm trajectories and this also requires calcium ions.

The present data show that the chemotactic response of siphonophore sperm, which requires millimolar concentrations of calcium ions, occurs through a non-transient induction of increased asymmetry of the flagellar waveform. It is proposed that the natural attractant operates to produce an increase in the intraaxonemal calcium concentration.

INTRODUCTION

Chemotaxis was recognized long ago for plant gametes (Pfeffer, 1884). In animals, fertilization and especially external fertilization has been assumed to involve random collision of gametes (Rothschild & Swann, 1951); chemotaxis between sperm and egg was described only in 1950 (Dan, 1950). Since then, chemotactic behaviour of spermatozoa has been found throughout the animal kingdom (see Miller, 1977, for a review). A common feature of the sperm response is a transient change in the flagellar beat pattern, which causes reorientation of the spermatozoon. In contrast to bacterial chemotaxis, which involves transitory methylation of membrane proteins (Kort, Goy, Larsen & Adler, 1975) and a rise in the intracellular cyclic GMP level (Hobson, Black & Adler, 1982), very little is known about signal transduction or the chemistry of attraction in spermatozoa.

In a previous paper (Carré & Sardet, 1981), the general features of fertilization in

siphonophores were described. Spermatozoa were shown to be attracted by an extracellular structure that is stuck on the egg surface, i.e. the cupule, and not to be attracted by an egg experimentally deprived of its cupule. The chemoattractant, which can be extracted from the cupule, is remarkably heat-stable, resistant to most proteases and does not induce the acrosome reaction. Preliminary observations indicate that the sperm movement is uniformly modified during the chemotactic response (Sardet *et al.* 1982) and that calcium ions are required for chemotaxis (Carré & Sardet, 1981; Cosson, Carré, Cosson & Sardet, 1983*b*). In this paper, sperm movement and its modulation by calcium ions have been analysed in the absence of attractant or in its presence, when distributed either uniformly or along a gradient. The siphonophore sperm response is discussed in terms of general sperm chemotactic behaviour.

MATERIALS AND METHODS

Materials

The siphonophores *Muggiaea kochi* and *Chelophyes appendiculata* were collected from April to June in the bay of Villefranche-sur-mer (France), with plankton nets pulled at depths of 0–50 m.

The sexual stages (= eudoxids), male or female, are liberated in the plankton. Male mature gonophores are identified by the opaque appearance of their manubrium. Female gonophores have a manubrium with 10–20 transparent eggs. For experiments, gametes were liberated by shredding the ectoderm of the manubrium and used within the next half hour.

To assay chemotaxis, cupules were isolated from eggs with tungsten needles (Carré & Sardet, 1981). They were used fresh or after fixation on a glass slide (air-dried followed by soaking in absolute ethanol). Fixed cupules are resistant to solubilization in low ionic strength buffers and are stable, by chemoattraction criteria, for at least 2 years, even when stored at room temperature. In contrast, fresh cupules or cupules air-dried but not soaked in absolute ethanol, are solubilized readily in distilled water, giving what we call 'cupule extract'. Usually, 30 manually isolated cupules were collected on a glass slide and covered by, at the most, 50 μl of distilled water. The cupules were dissolved instantaneously and the resulting solution was either mixed with 1 % agar in sea water and forced into a capillary pipette, or diluted in a sperm suspension.

Calcium-free sea water contained 516 mM-NaCl, 10 mM-KCl, 34 mM-$MgCl_2$, 20 mM-$MgSO_4$ and 10 mM-Tris·HCl (pH 8·2). NaCl and KCl were Carlo Erba products, magnesium salts and Tris were from Merck. A23187 was purchased from Lilly Research Laboratories and kept as a stock solution at 1 mg/ml in dimethylsulphoxide.

Recordings of sperm movement

The sperm was placed in a drop of sea water between a glass slide and a coverglass, both of which were coated with bovine serum albumin. Sperm motility was observed using stroboscopic illumination (Chadwick-Helmut Strobex) and dark-field optics (Zeiss low-power dark-field condenser). Photomicrographs were taken on Ilford HP5 films with continuous flashing of the illuminator and exposure times set by the shutter. Low-power photomicrographs were made with a Zeiss Plan 16/0·35 objective and a flash frequency of 20 Hz giving one exposure for every other beat of the flagellum. Higher-resolution photomicrographs were made with a Zeiss Plan 25/0·45 objective and flash frequencies of 30 or 40 Hz.

Flagellar beat frequencies were measured by reference to the calibrated frequency of the flash illuminator and values were averaged on 20 spermatozoa from five different fields. Measurements of sperm velocity were made either on printed enlargements of the negatives or directly under the enlarger. In both cases, distances were evaluated by comparison with a calibrated scale. The velocity of the spermatozoa was calculated by measuring the distance travelled during the exposure or during some multiples of the flash illuminator frequency and was expressed either in $\mu m\,s^{-1}$ or in μm per beat, i.e. propulsive efficiency (Cosson, Tang & Gibbons, 1983*a*). Curvatures of trajectories were

measured by fitting the recorded arcs of trajectories with the calibrated circle of comparable radius, and are expressed either as angular deviation per time unit or as angular deviation per beat, i.e. turning rate (Brokaw, Josslin & Bobrow, 1974).

When prolonged recordings of sperm trajectories were required, sequences of 1 min to 15 min of sperm movement were shot under permanent illumination using videotape equipment. The movie camera (National TV camera) had a constant frame-rate of 50 Hz, and was connected to a National time-lapse UTR videoscope. Individual sperm traces were drawn from the TV screen by plotting the head positions every four frames. Velocities and paths curvatures were measured on successive portions of the trajectories as described above.

RESULTS

Behaviour of spermatozoa in the absence of chemoattractant

When the manubrium is disrupted, spermatozoa from siphonophores (*M. kochi* and *C. appendiculata*) swim out as a cloud and disperse very quickly. This prompt dispersion upon dilution is the result of the instantaneous initiation of an efficient propulsive movement with nearly straight trajectories. When swimming freely in a drop of sea water, the spermatozoa move in spirals along nearly straight trajectories until they reach a glass surface, where they become trapped. Trapped spermatozoa swim in circular trajectories parallel to the surface and, because they remain in one plane, these are the cells that we observed during our experiments.

Sperm movement in natural sea water. M. kochi spermatozoa describe circular trajectories with average diameters of 700 μm at an average velocity of 250 $\mu m\,s^{-1}$ (Fig. 1). Analysis of 132 independent trajectories shows that their diameters mostly range from 600 μm to 800 μm, with 13 % having a diameter smaller than 600 μm and 11 % larger than 800 μm. A less-extensive analysis performed on *C. appendiculata* spermatozoa gives values of 1000–1500 μm for the diameters of their trajectories. Sperm velocities in both species vary from 200 $\mu m\,s^{-1}$ to 400 $\mu m\,s^{-1}$ with beat frequencies of the sperm flagellum of 35–40 Hz.

Combining velocity and beat frequency values leads to a calculated propulsive efficiency of 6·2 μm/beat. From the size of the trajectories, one can estimate that it takes about 350 beats for a spermatozoon to complete a circle. This means that, at every beat, the angular deviation of the sperm head is 0·018 radians. This value, which is smaller than those measured for live sea-urchin spermatozoa (0·1 radian/beat: Cosson *et al.* 1983*a*), reflects the symmetry of the waveform of the siphonophore sperm flagella (Fig. 1B, C). The flagella propagate planar waves with similar bend-angle values for direct and reverse waves. Within a sperm suspension or within freshly shed preparations of mature spermatozoa, the values of these parameters are highly reproducible (beat frequency of 38–40 Hz, propulsive efficiency of 6 μm/beat and turning rate of 0·018 radians/beat). Furthermore, observations on individual spermatozoa for longer periods of time (5–10 min), show that this symmetrical and efficient movement is repeated uniformly, without detectable transient modifications such as abrupt turns or transient quiescence. However, with aging of a preparation, the sperm velocity and flagellar beat frequency decrease quickly. In contrast, non-mature spermatozoa never propagate waves of higher frequency than 1–2 Hz, and these are insufficient for propulsion.

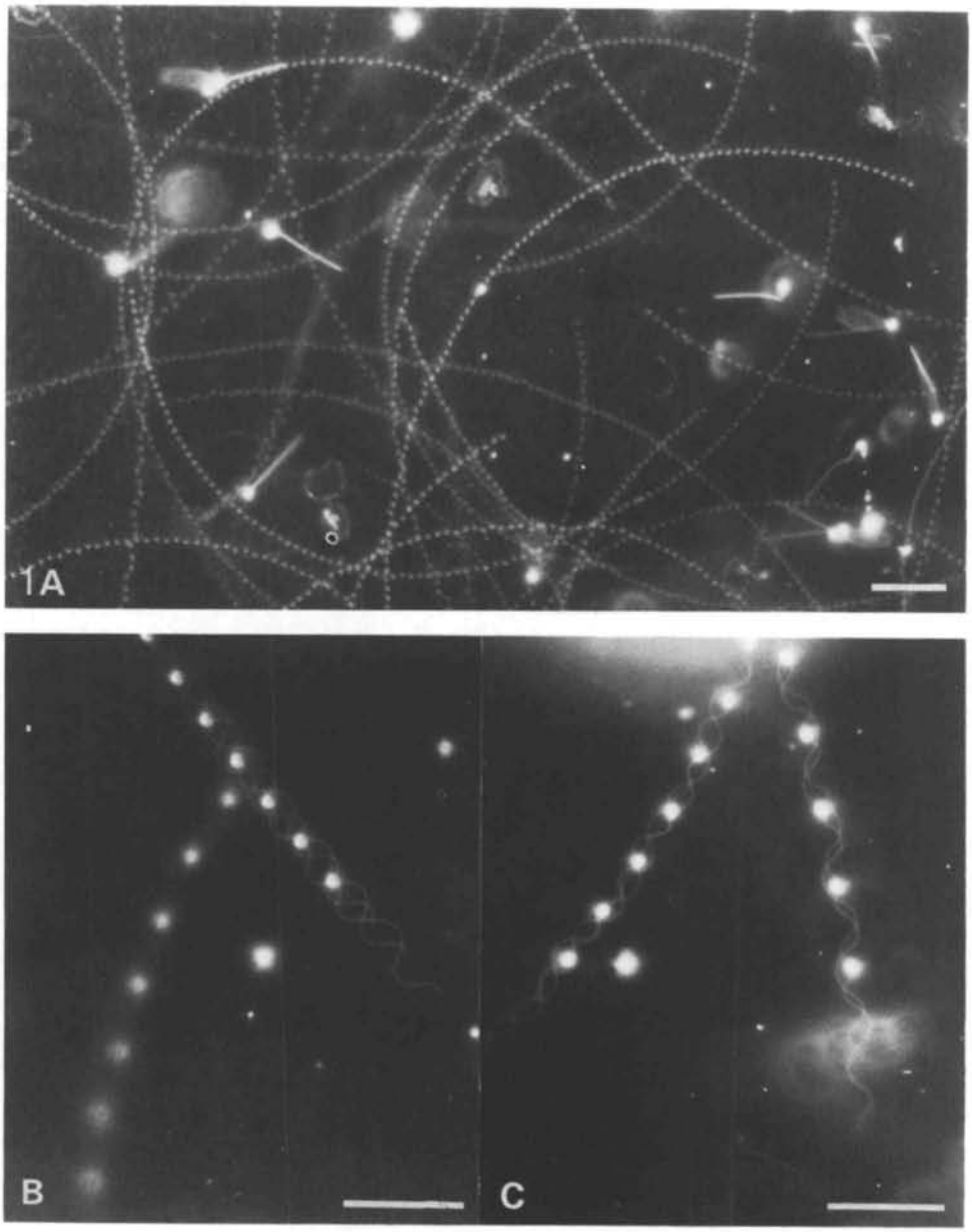

Fig. 1. Dark-field microphotographs of tracks made by the heads of native spermatozoa, swimming in natural sea water at the surface of a bovine serum albumin-coated glass slide. A. *M. kochi* spermatozoa. Flash frequency, 30 Hz; exposure time, 5 s. Bar, 50 μm. B, C. *C. appendiculata* spermatozoa. Flash frequency, 10 Hz; exposure time, 1 s. Bar, 50 μm.

Sperm movement in modified sea water. Thus, the major difference between sea-urchin and siphonophore sperm movement lies in the turning-rate values. Considering the well-documented control exerted by Ca^{2+} on that parameter in sea-urchin sperm movement (Brokaw *et al.* 1974; Gibbons & Gibbons, 1980), the question arises

Table 1. *Parameters of sperm movement in the presence or the absence of calcium ions in sea water*

Species	Calcium concentration (M)	Sperm velocity ($\mu m\ s^{-1}$)	Diameters of trajectories (μm)	Flagellar beat frequency (Hz)	Turning rate (radian/beat)	Propulsive efficiency (μm/beat)
M. kochi	$1{\cdot}1 \times 10^{-2}$	412 ± 40	760 ± 280	39 ± 2	0·086	10·5
	$<10^{-6}$	178 ± 15	560 ± 140	$35{\cdot}3 \pm 2{\cdot}6$	0·055	5
C. appendiculata	$1{\cdot}1 \times 10^{-2}$	263 ± 31	1524 ± 420	$37 \pm 1{\cdot}1$	0·03	7·1
	$<10^{-6}$	159 ± 19	770 ± 470	$34{\cdot}8 \pm 3$	0·036	4·5

Records of the swimming behaviour of the spermatozoa from both species were obtained and analysed as described in Materials and Methods. Sea water containing $1{\cdot}1 \times 10^{-2}$ M-calcium is either natural sea water or artificial sea water supplemented with calcium. Ca^{2+}-free sea water is estimated to contain less than 10^{-6} M-calcium, from the composition of the salts utilized. Measurements were done on 25 and 42 trajectories for *M. kochi*, and 16 and 15 for *C. appendiculata*.

as to whether the symmetrical beat movement of siphonophore sperm is related to a low concentration of intraflagellar Ca^{2+}.

As shown in Table 1, there are some differences between the parameters of siphonophore sperm movement in natural sea water (calcium concentration: $1{\cdot}1 \times 10^{-2}$ M) and in Ca^{2+}-free sea water (artificial sea water made without calcium ions, which leads to less than 10^{-6} M-Ca^{2+}). Decreases in sperm velocity and in the diameter of their trajectories are observed. This is probably due to a loss of sperm viability in the absence of calcium ions. Indeed, with *C. appendiculata* and *M. kochi* sperm, we are unable to observe any sperm movement in sea water with concentrations above 0·1 mM-EGTA.

However, ability of the calcium to regulate sperm movement is supported by experiments using a calcium ionophore. When A23187 is added to natural sea water, the trajectories described by the spermatozoa have smaller diameters and this effect increases with the concentration (Cosson *et al.* 1983*b*). In artificial sea water, the reduction in the size of the swimming paths induced by A23187 is dependent on the concentration of calcium ions: the trajectories show a fourfold reduction in their diameters in natural sea water (10^{-2} M-calcium) against a 1·5-fold reduction in 10^{-6} M-Ca^{2+} (Fig. 2; Table 2). This corresponds, for A23187-treated spermatozoa, to a progressive increase in the diameter of their trajectories with decreasing calcium concentrations.

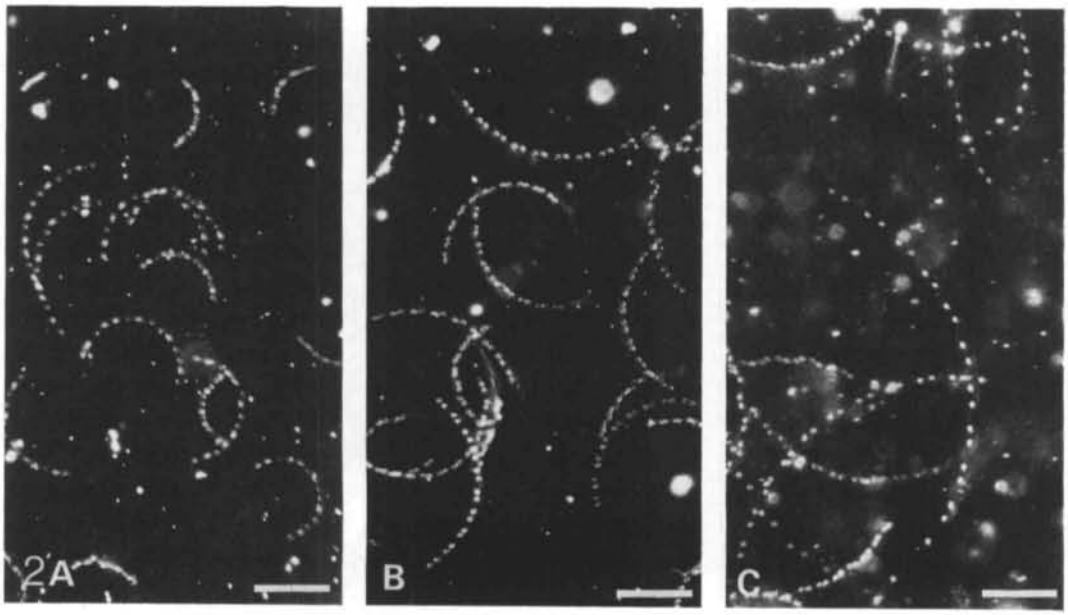

Fig. 2. Effect of calcium ion concentration on the swimming behaviour of A23187-treated spermatozoa. Sperm were collected in Ca^{2+}-free sea water and were subsequently diluted in artificial sea water containing 2·5 μg ml^{-1} of A23187 and 11 mM-$CaCl_2$ (A), 0·1 mM-$CaCl_2$ (B) and no added $CaCl_2$ (C). Records of sperm trajectories started within 1 min. Exposure time, 1 s; flash frequency, 40 Hz (A), 20 Hz (B–C). Bar, 50 μm.

Table 2. *Modification of A23187-treated sperm movement by varying the calcium ion concentration*

Calcium concn (M)	Sperm trajectories diameter (μm)		Sperm velocity (μm s^{-1})	
	Non-treated sperm	A23187-treated sperm	Non-treated sperm	A23187-treated sperm
$1{\cdot}1 \times 10^{-2}$	805 ± 314	204 ± 61	235 ± 24	248 ± 76
10^{-4}	720 ± 200	327 ± 72	240 ± 36	113 ± 36
$<10^{-6}$	566 ± 144	372 ± 145	178 ± 15	174 ± 29

Sperm trajectories were analysed and recorded as described in Materials and Methods. Ca^{2+}-free sea water was supplemented with Ca^{2+} to the desired concentration.

Behaviour of spermatozoa in the presence of attractant sources

In the presence of eggs. When swimming freely in natural sea water, mature spermatozoa sometimes arrive in the vicinity of an egg. Carré & Sardet (1981) observed that the sperm accumulate, less than 1 min after their introduction, around an extracellular structure of the egg called the cupule. Observations on spermatozoa swimming within 2 μm of the egg show that the sperm move in smaller circles when they are close to the cupule than when they are further away. This change in their behaviour appears uniformly throughout the population of sperm cells (Fig. 3). Occasionally, chemotactic turns similar to those described by Miller & Brokaw (1970) are observed, but they are unusual (0·8 % of the 660 sperm trajectories analysed). As this behaviour is observed infrequently it cannot be considered the major chemotactic response of the spermatozoa.

After fertilization, the extrusion of a viscous liquid precedes and probably causes the detachment of the cupule. Subsequently, the cloud of attracted spermatozoa is carried away from the egg surface with the cupule. When fertilization tests are performed in a micro-chamber detachment of the cupule from the egg is prevented. In these cases, the chemotactic competence of the cupule is observed even during segmentation (Fig. 3). Evidently, the liberation of the attractant is not inhibited after fertilization in siphonophores, in contrast to the observations of Freeman & Miller (1982) on other Hydrozoa.

In the presence of isolated cupules. Cupules were isolated, dried and fixed in absolute ethanol as described in Materials and Methods. This treatment probably precipitates some protein components of the structure. It prevents the cupule, which appears bright in dark-field microscopy, from further solubilization when rehydrated, and traps the attractant, which then diffuses out slowly. Indeed, fixed cupules continue to attract sperm after more than 100 h of soaking in natural sea water or after 2 years in dry storage. Such preparations allow one to record and analyse the chemotactic response of the spermatozoa in conditions of planar movement (Fig. 4). Observations on sperm trajectories confirm that the spermatozoa describe circles of smaller

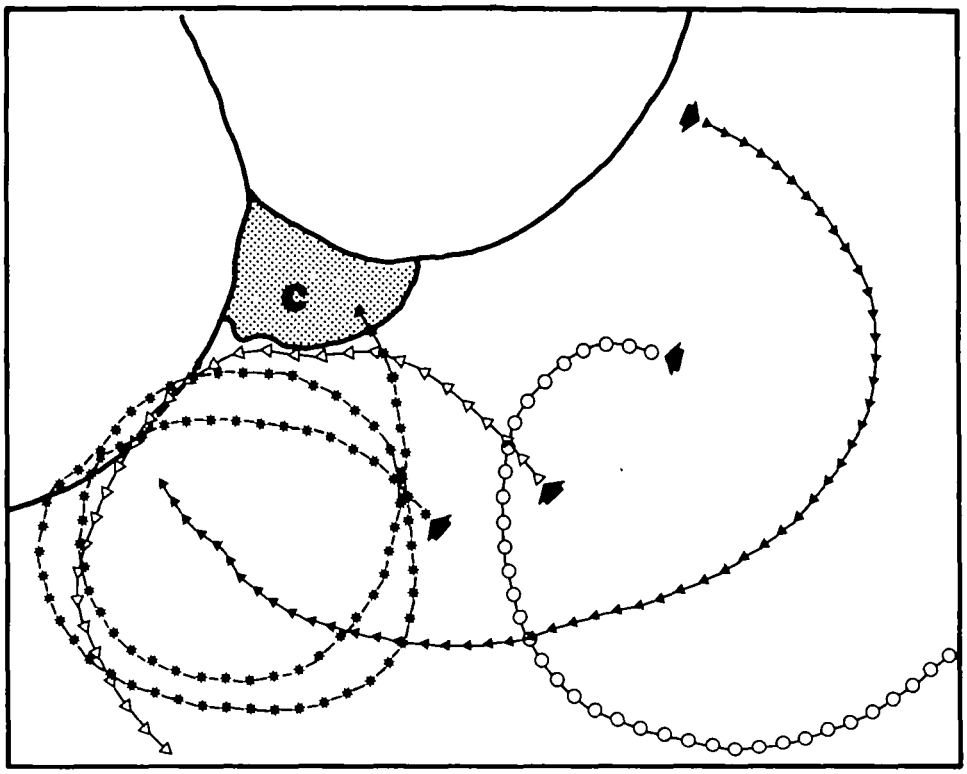

Fig. 3. Maintenance of sperm chemotaxis towards the cupule (c) after the segmentation of the egg. *M. kochi* egg and spermatozoa were set in a microchamber in natural sea water and sperm trajectories were recorded by time-lapse video. The tracings are from a sequence after the first segmentation and positions of sperm heads are indicated every 100 ms. Arrows indicate the direction of sperm displacement.

size near the cupule, around which they turn with rather uniform curvature. The diameter of the swimming paths decreases threefold from beyond 700 μm to within 200 μm on the cupule (Table 3). The diameter of the trajectories decreases progressively at distances from 5·5–2·3 mm from the cupule and more quickly within 1 mm of the cupule. This behaviour is compatible with the dilution of an active compound diffusing from the cupule.

Neither the beat frequency of the flagella nor the velocity of the spermatozoa circling the cupule are different from those of the spermatozoa swimming in the absence of chemoattractant. Thus, the modification of the swimming behaviour of

Fig. 4. Sperm chemotaxis towards an isolated cupule (c). Spermatozoa, collected in natural sea water were deposited near a cupule and trajectories were recorded by dark-field microscopy as described in Materials and Methods. A. Spermatozoa and cupule from *C. appendiculata*. Flash frequency, 25 Hz; exposure time, 1 s. Bar, 50 μm. B. Spermatozoa and cupule from *C. appendiculata*. The contrast of the cupule has been improved by soaking it in sea water containing 1 mg ml^{-1} of bovine serum albumin before fixation in absolute ethanol. Flash frequency, 10 Hz; exposure time, 1 s. Bar, 50 μm. C. Flash exposure of spermatozoa from *M. kochi* near a cupule. Bar, 20 μm.

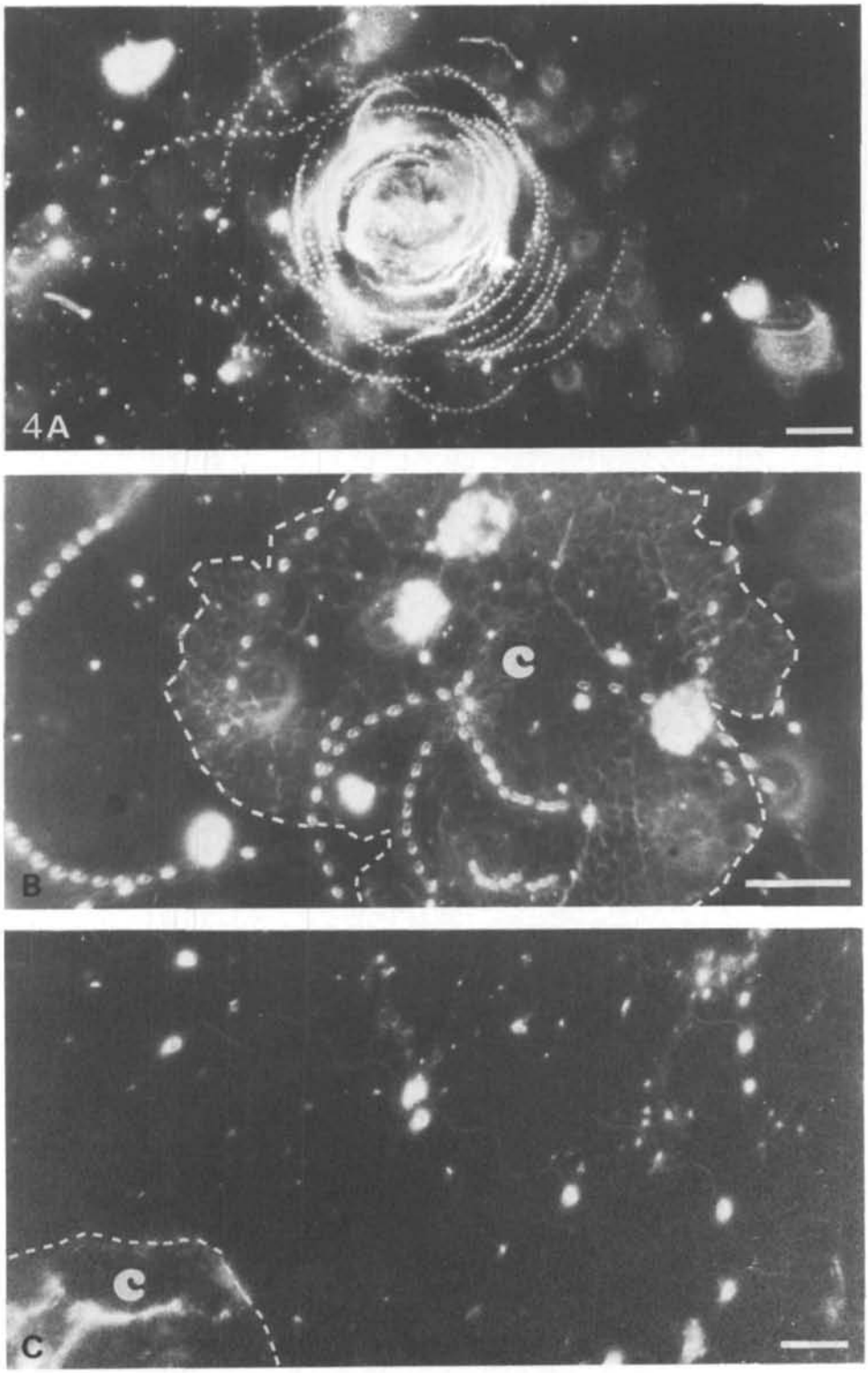

Fig. 4

Table 3. *Progressive increase in flagellar asymmetry on approaching a cupule*

Distance to the cupule (mm)	No. of trajectories analysed	Diameter of the trajectories (μm)	Sperm velocity ($\mu m\ s^{-1}$)	Flagellar beat frequency (Hz)
On the cupule	19	188 ± 53	360 ± 37	41 ± 0·5
0·2	26	291 ± 150	375 ± 51	41 ± 0·6
1·3	17	436 ± 159	341 ± 34	38·6 ± 1·4
2·3	15	581 ± 230	371 ± 50	36·4 ± 3
3·3	22	575 ± 208	416 ± 80	37·5 ± 1·6
5·5	19	726 ± 243	383 ± 54	N.D.
In the absence of attractant	25	760 ± 286	412 ± 40	38·9 ± 2·3

Trajectories of sperm swimming in natural sea water were recorded and analysed as described in Materials and Methods. Distances from the cupule were calculated from the coordinates on the calibrated stage of the microscope, both for the cupule and the field of spermatozoa that were observed. N.D., not determined.

siphonophore sperm during a chemotactic response is a 'constant frequency response', like that observed with sea-urchin spermatozoa, where calcium ions act as a natural effector (Gibbons & Gibbons, 1980).

In the presence of cupule extracts. Cupule extracts can be included in a pipette of agar as described in Materials and Methods. One presumes that diffusion generates a gradient of the attractant at the end of the pipette that is not unlike the natural situation (Fig. 5). This experimental arrangement allows the recording of sperm trajectories as they approach the pipette just after its introduction into the observation chamber. In Fig. 5, the trajectories recorded 7–8 s after the introduction of the pipette (traces 1–3) are similar to those of spermatozoa swimming in the absence of attractant. Traces 5–6 (+12 s and +18 s) are characteristic of attracted behaviour. The chemotactic response usually develops within 10 s.

Solubilized cupules also offer the possibility of observing sperm behaviour in the presence of a uniform concentration of attractant in sea water. Such experiments show that sperm trajectories become uniformly smaller in diameter immediately after addition of the chemoattractant (Fig. 6). Preliminary observations suggest that the reduction in the diameter of the trajectories is related to the dilution of the attractant; but as high concentrations of attractant severely reduce sperm viability, this proportionality could not be established.

Calcium requirement for the chemotactic response

The curvature of the sperm trajectories is the only feature of sperm behaviour that is modified near a source of attractant. The calcium ionophore A23187 has a similar effect. This suggests that the chemotactic response might be mediated by a modification of the intraflagellar calcium concentration. Therefore, chemotaxis was tested in artificial sea water lacking calcium ions.

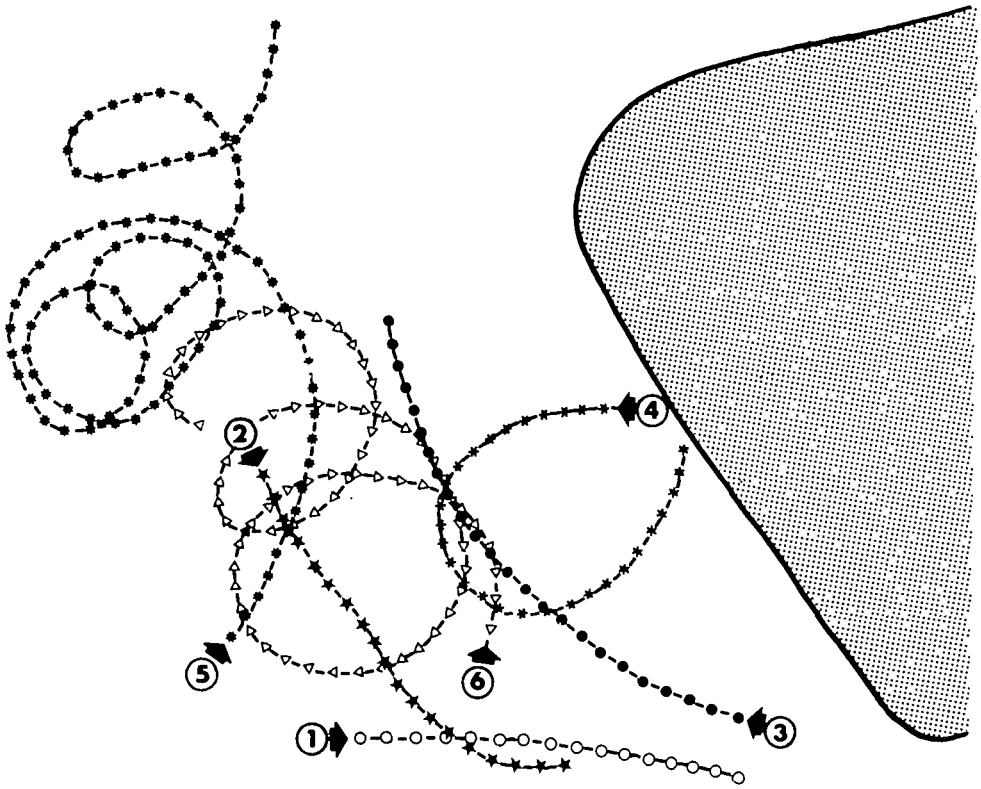

Fig. 5. Modification of sperm trajectories following the introduction of a pipette containing attractant. The active fraction solubilized from the cupules was prepared and forced into the pipette as described in Materials and Methods. Recordings were started from the time of introduction of the pipette into the sperm suspension and the numbers refer to tracks of spermatozoa arriving successively in the vicinity of the pipette. Track 1 started 3 s after the introduction of the pipette; 2, 4·5 s; 3, 7·5 s; 4, 10 s; 5, 12 s; 6, 18 s. Positions of sperm heads are indicated every 0·1 s. Arrows show the direction of sperm movement. The stippled area represents the tip of the pipette.

In Ca^{2+}-free sea water the cupules detach more easily from the egg surface. No sperm accumulation is seen around these cupules. Similarly, cupules manually isolated and fixed in ethanol are not surrounded by circling spermatozoa in the absence of calcium, whatever sperm concentration is used for the test (Fig. 7). Indeed, they cross the cupule area with trajectories whose curvatures approach those in natural sea water, and the waveforms of their flagella are nearly symmetrical (Fig. 7B, C). Adding calcium back to the medium results in the formation of a sperm cloud around the cupule (Fig. 7D, E) due to the decrease in the diameters of the circles described by the sperm cells (Table 4). This restoration of chemotaxis does not happen in the presence of calcium concentrations lower than 0·1 mM, appears partially between 0·1 mM and 1 mM-Ca^{2+} and is fully expressed above 5–6 mM-Ca^{2+}

Calcium is also required for the sperm response towards cupule extracts included in a pipette. Comparison of the data in Figs 5 and 8 demonstrates this requirement.

Experiments with a uniform concentration of attractant in Ca^{2+}-free sea water show

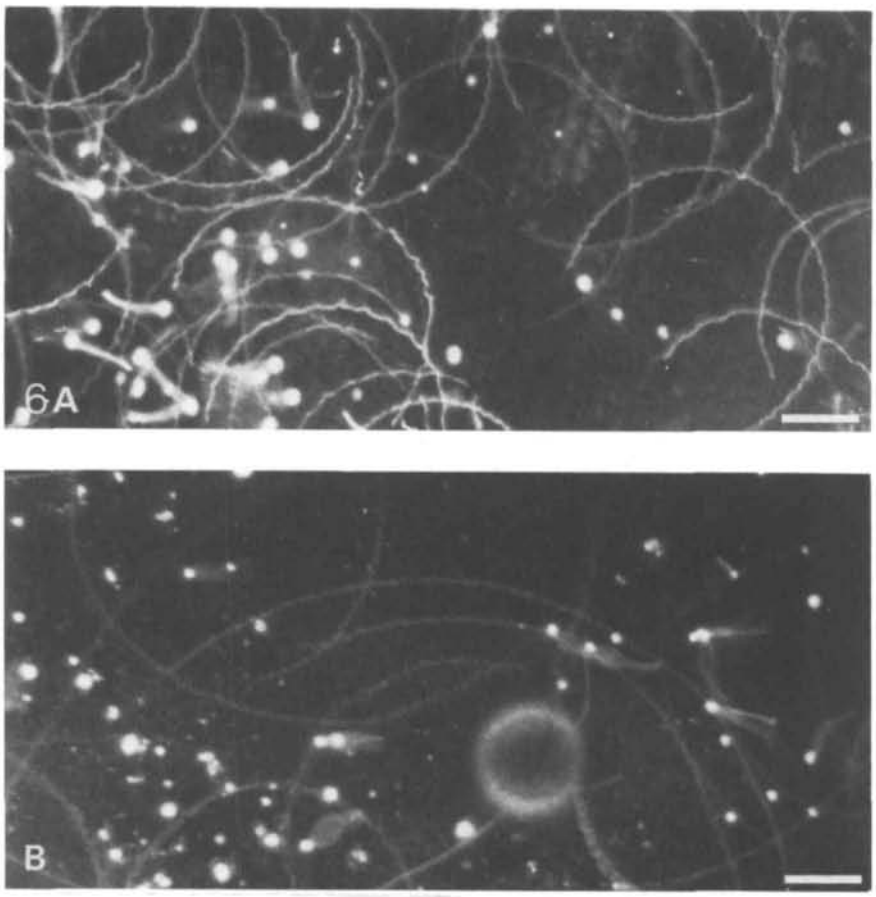

Fig. 6. Modulation of sperm movement by solubilized attractant in natural sea water (A) and in Ca^{2+}-free sea water (B). The active fraction was prepared as described in Materials and Methods and diluted 100 times in the sperm suspension. Sperm movement was recorded at 30 Hz flash frequency, using a 10-s exposure. Bar, 50 μm.

trajectories 323 μm in diameter; in natural sea water they are 213 μm in diameter. This illustrates further the calcium-dependence of the attractant-induced regulation of swimming behaviour, and compares closely with the modulation of sperm trajectories upon treatment with A23187 (Table 2). However, in Ca^{2+}-free sea water, the diameters of the trajectories of treated spermatozoa were smaller than those of native spermatozoa. This effect suggests that the residual calcium ions in our assays (less

Fig. 7. Dependence of chemotaxis on the presence of calcium ions. Spermatozoa from *M. kochi* were deposited near an isolated cupule (C) in Ca^{2+}-free sea water. Sperm distribution was found to be homogeneous throughout the preparation (A) and no modification of the curvatures of sperm trajectories was observed (B–C). Flash frequencies and exposure times were, respectively: A, 30 Hz, 5 s; B, 40 Hz, 0·25 s; C, 20 Hz, 1 s. Addition of 10 mM-$CaCl_2$ to the assay restored curved sperm trajectories centred on the cupule (D–E). Flash frequencies, 20 Hz (D) and 50 Hz (E); exposure times, 1 s (D) and 15 s (E). Bar, 50 μm, except B (20 μm).

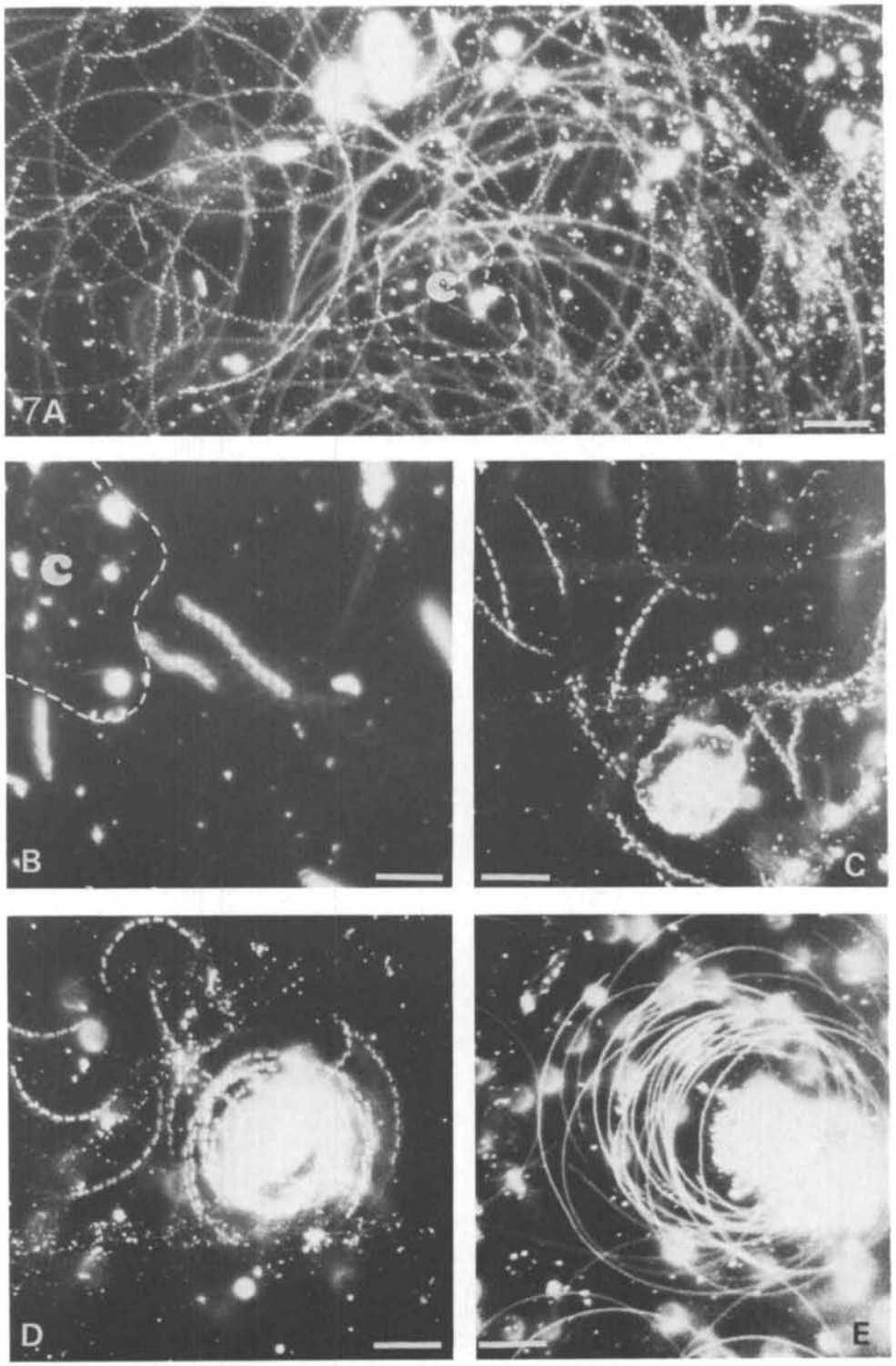

Fig. 7

Table 4. *Recovery of sperm chemotaxis upon addition of calcium*

Calcium ions in artificial sea water	Distances to the cupule (mm)	Diameters of the sperm trajectories (μm)
Less than 10^{-6} M	On the cupule	410 ± 137
Less than 10^{-6} M	More than 6	481 ± 67
6 mM	On the cupule	195 ± 60
6 mM	1	326 ± 108
6 mM	2	372 ± 132

Spermatozoa, collected in Ca^{2+}-free sea water, were deposited near a cupule. Lack of chemotactic response was observed and sperm movement was recorded either on the cupule or far from it. Upon addition of 6 mM-calcium ions, chemotaxis was fully restored and recorded at different distances from the cupule.

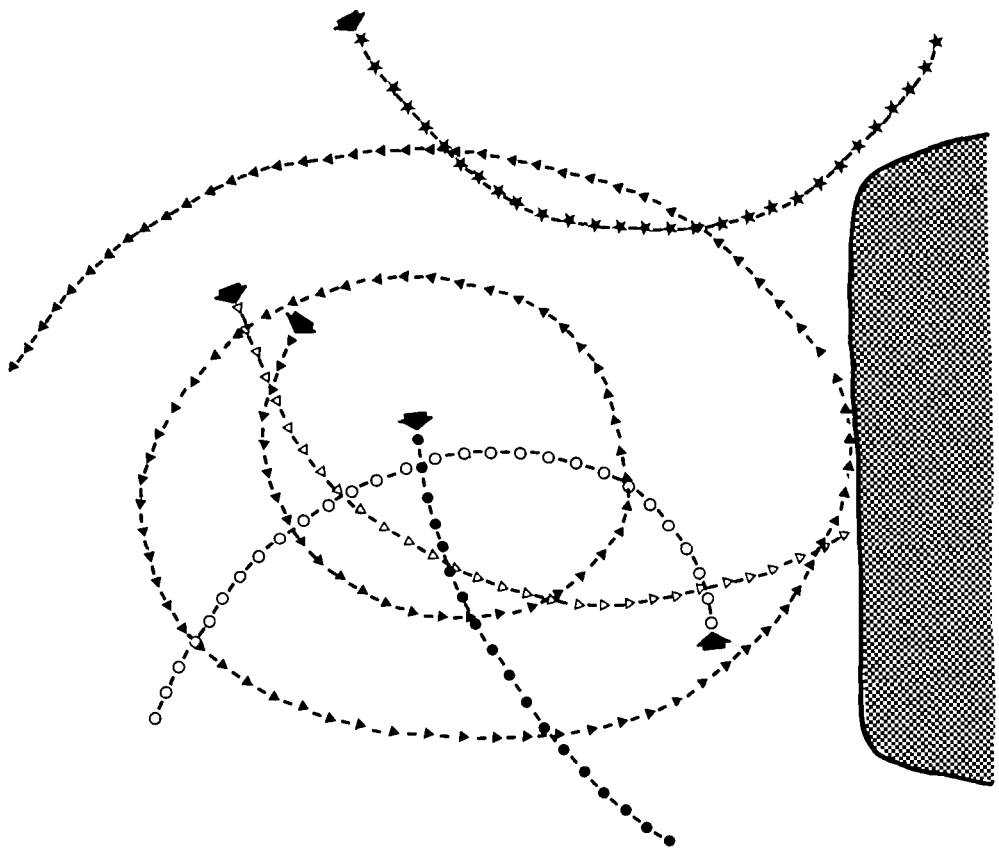

Fig. 8. Sperm trajectories near a pipette of attractant in Ca^{2+}-free sea water. Experimental conditions were as for Fig. 5, except for the absence of added $CaCl_2$ in artificial sea water. Head positions are indicated every 0·1 s. Arrows indicate the direction of sperm movement.

than 10^{-6} M) might be sufficient to allow a calcium influx when the permeability is artificially increased by A23187 or by the attractant and, therefore, that the axonemal calcium concentration might normally be less than 10^{-6} M. As already stated, lowering the calcium concentration in artificial sea water, with 1 mM-EGTA, stops sperm movement altogether. This prevents a more precise estimation of the intraflagellar calcium concentration.

In an attempt to compare the function of the natural attractant with that of the ionophore A23187, competition between the two was investigated. In natural sea

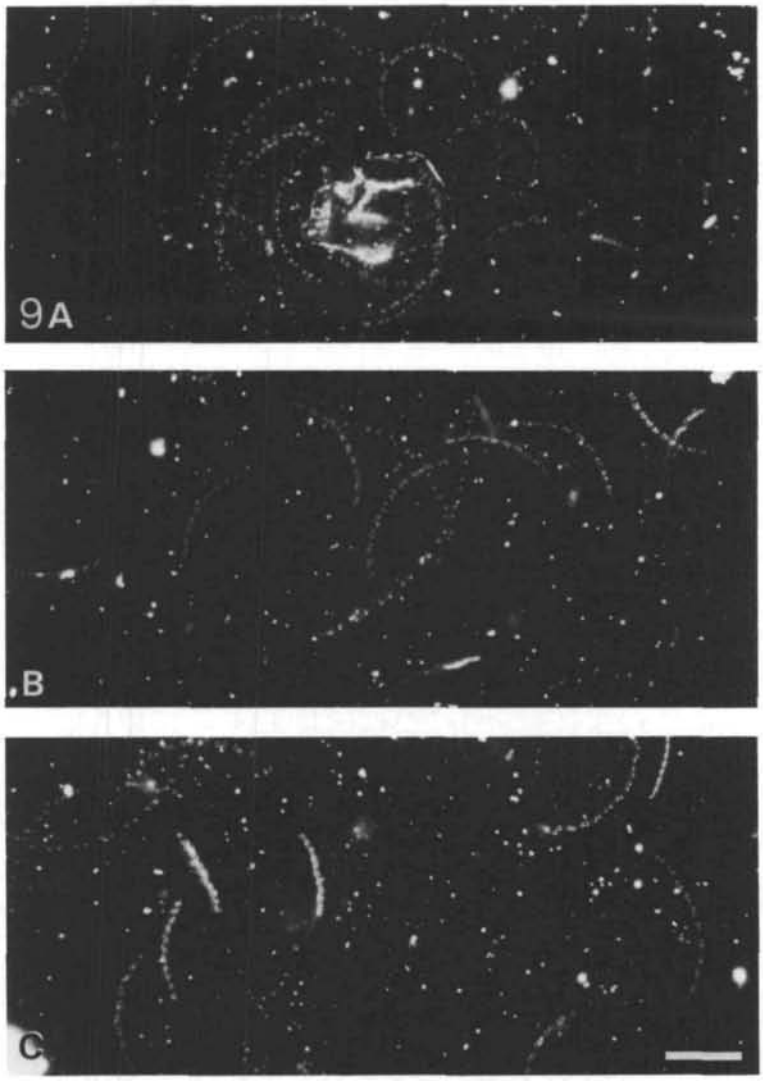

Fig. 9. Response of *M. kochi* spermatozoa to the presence of a cupule in natural sea water containing 2·5 μg ml^{-1} of A23187. Experimental procedure was as for Fig. 4, except for the presence of A23187 in sea water. Sperm tracks were successively recorded on the cupule (A), at 0·1 mm (B) and at 0·3 mm from the cupule (C). Flash frequencies, 20 Hz; exposure times, 1 s. Bar, 50 μm.

water and in the presence of A23187, the sperm response towards a cupule is severely decreased (Fig. 9). The curvature of sperm trajectories are of $2{\cdot}0 \pm 0{\cdot}5$ radian s^{-1} on the cupule against $1{\cdot}6 \pm 0{\cdot}3$ radian s^{-1} at 0·1 and 0·3 mm from it. The average number of sperm cells present per field is, respectively, 36 %, 34 % and 29 %. Consequently, this behaviour does not allow the establishment of a cloud of spermatozoa on the cupule. However, around the cupule, a few trajectories are narrower than the average ones and they tend to be centred on it. This would suggest the persistence of a residual chemotactic response, but a definite conclusion on competition cannot be drawn as the attractant is a very efficient modulator of sperm movement and as the A23187 concentration cannot be varied on a large scale. Attempts to induce a chemotactic response of spermatozoa towards a pipette containing A23187 did not succeed. Nevertheless, with the exclusion of the chemotactic behaviour of a few spermatozoa circling close to the cupule, the present results demonstrate the lack of a cloud formation in the cupule area in the presence of A23187.

DISCUSSION

A common feature of sperm chemotactic behaviour involves a transient response of the cells, resulting in their precise detection of a gradient of attractant and a resumption of propulsive swimming precisely directed towards the attractant source (Miller & Brokaw, 1970; Miller, 1982). The transient change in the direction of sperm movement has been shown to be the result of alterations in the symmetry of flagellar patterns, inducing loopings (Miller & Brokaw, 1970). Such sudden turns are observed when the spermatozoa are swimming across the concentration gradient and this suggests that changes in chemoattractant concentration might trigger a turning mechanism. However, this hypothesis of a 'sampling mechanism' (Miller & Brokaw, 1970) has not been supported experimentally.

We describe here a somewhat different form of chemotactic response displayed by spermatozoa of siphonophores, and studied in two species (*M. kochi* and *C. appendiculata*). The general features of *M. kochi* reproduction have been described previously (Carré & Sardet, 1981; Sardet *et al.* 1982).

Spermatozoa from *M. kochi* or from *C. appendiculata* have a uniform movement: they describe circular trajectories of large diameters (700–1000 μm) at velocities from 200 $\mu m\, s^{-1}$ to 400 $\mu m\, s^{-1}$, propelled by flagella beating at 35–40 Hz. Usually, more than 95 % of the sperm cells are motile. When approaching a source of attractant (native cupule stuck on the egg, native cupule isolated from the egg, fixed cupule stuck on a glass slide, or pipette containing the attractant trapped in agar), the spermatozoa show a progressive increase in the curvature of their trajectories. There are no sudden modifications that lead to a precise reorientation of their trajectories. The spermatozoa approach the attractant source through several helicoidal arcs of decreasing radius and swim around it in ever tighter circles, without becoming aggregated at the point of liberation of the attractant. After 5–10 min more than 99 % of the spermatozoa are describing circular trajectories of about 200 μm in diameter, roughly centred on the source of attractant. Moreover, the extracellular compartmentation

of the active substance allows extraction of a crude sample, which permits sperm behaviour to be analysed in the presence of a homogeneous distribution of the attractant. This characteristic feature of siphonophore sperm chemotaxis – uniform switching from one type of movement to another – makes this system particularly suitable for further biochemical analysis of the transduction mechanism (work in progress).

The attractant reduces the diameter of the sperm trajectories without modifying either sperm velocity or flagellar beat frequency. Similar modulations of flagellar movement can be obtained by manipulation of the calcium concentration in Triton-demembranated sea-urchin spermatozoa (Brokaw *et al.* 1974) or in the flagellar apparatus isolated from *Chlamydomonas* (Hyams & Borisy, 1978). In native siphonophore spermatozoa, such control is demonstrated by altering the concentration of exogenous calcium in the presence of the ionophore A23187 (present work; Cosson *et al.* 1983*b*). For the chemotactic response of siphonophore spermatozoa to cupules or pipettes containing the attractant a concentration of more than 0·1 mM-calcium is required, and higher concentrations are required in the presence of a uniform distribution of the attractant. Moreover, sperm chemotaxis is considerably decreased when A23187 is present in the assay. This indicates that siphonophore sperm movement is susceptible to modulation by calcium ions, and that the function of the attractant might be to increase the intra-axonemal calcium concentration. On the basis of the high symmetry of flagellar waveform of native spermatozoa, it is suggested that the calcium concentration inside the flagella might be lower than 10^{-6} M in the absence of attractant. This low concentration might be maintained by an outward pump (Doughty, 1978). It is proposed that, when a spermatozoon arrives in an attractant gradient the binding of the attractant to the sperm cells induces a calcium influx that is related to the concentration of attractant, and consequently produces an increase in the asymmetry of the flagellar beat pattern. Helicoidal approaches of spermatozoa to the attractant source would be generated by the superimposition of this effect on the constant pumping of calcium ions at all times. It should be possible to test the validity of this model for siphonophore sperm chemotaxis by more precise analysis of the curvature along sperm trajectories (work in progress).

The question remains as to whether 'chemotaxis' is an appropriate description of the sperm responses we have observed. If the term chemotaxis is to be used *sensu stricto*, that is, movement towards a chemical source, whatever type of movement is generated, then the siphonophore sperm behaviour should qualify as chemotaxis. Siphonophore eggs contain a compound that diffuses slowly from the cupule and influences sperm movement in such a way that a large accumulation of sperm cells around the cupule is produced. However, a considerable body of data describing chemotactic behaviour in organisms as different as bacteria and spermatozoa lead to a different conception of chemotaxis based on a succession of periods of smooth or regular movement interrupted by short periods of reorientation (Adler, 1976; Miller, 1977). This mechanism requires sperm cells to be able to detect rapid changes in concentration of attractant and, more importantly, to transform the directional

information in the gradient into a precise reorientation of their movement. Such behaviour is definitely not associated with sperm accumulation near the cupule in siphonophores.

We have no indication whether the attractant is acting by liberating calcium ions stored in some sperm compartment or whether it triggers a calcium influx. The calcium requirement for sperm chemotaxis has been demonstrated already (Brokaw, 1974; Miller, 1975; Carré & Sardet, 1981), and influxes of calcium ions have been implicated in the tactile behaviour of unicellular organisms (Naitoh & Kaneko, 1972; Holwill & MacGregor, 1976). However, no clear evidence is available concerning the origin of the calcium ions acting as transducers.

Recent observations on sperm chemotaxis in ascidians and its calcium requirement lead Miller to question the localization of the site of calcium entry in the axonemes (Miller, 1982). In *Paramecium* the ciliary membrane is differentiated at its proximal end into a ring of granule plaques, which have been found associated with calcium channels (Plattner, 1975). Freeze-etch studies of siphonophore sperm membranes have revealed the presence of such a necklace structure at the base of the flagella (Sardet *et al.* 1982). Considering that the flagella are anchored on the sperm head through a large pericentriolar complex and a long striated rootlet (Carré, 1979, 1984), and that calcium-induced contraction of the striated flagellar rootlet has been reported in *Chlamydomonas* (Salisbury & Floyd, 1978), one can propose the following sequence as a possible mechanism of activation of asymmetry during the chemotactic response of siphonophore spermatozoa: binding of the attractant on the sperm membrane, subsequent openings of calcium channels located in the differentiated proximal part of flagellar membrane; calcium influxes into the axoneme near the pericentriolar complex and associated structures; contraction of the striated rootlet with subsequent asymmetric translation of diametrically opposed flagellar microtubules. Support for this model will have to await the demonstration of calcium-induced contraction of the rootlet and a fine-structural analysis of the striated root positions during the chemotactic response, both data that might be obtainable from siphonophore spermatozoa.

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