BIOLOGY OF REPRODUCTION 36, 1191–1198 (1987)

Initiation of Hyperactivated Flagellar Bending in Mouse Sperm within the Female Reproductive Tract[1]

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ABSTRACT

To determine where and when hyperactivation is initiated in vivo, the flagellar curvature ratios (fcr) of mouse sperm within the female reproductive tract were measured from videotape recordings and compared with those of epididymal sperm incubated under capacitating conditions in vitro. The fcrs and linearities of trajectory were significantly lowered after 90 min of incubation in vitro, indicating that hyperactivation had been initiated by that time. The flagellar curvature ratios of sperm at the colliculus tubarius, within the uterotubal junction, and in the isthmus, measured at 1–2 h postcoitus and approximately 1 h before and 1 h after ovulation, were found to have fcrs that were not different from those of sperm incubated for 90 min in vitro. It was concluded that the tract sperm had initiated hyperactivated flagellar bending before the time of ovulation and before entering the oviduct. Only sperm in the lower isthmus 1 h before ovulation had fcrs that were significantly different from sperm incubated for 90 min in vitro, but not from sperm measured at the beginning of incubation in vitro. This could be the result of motility suppression in the lower isthmus.

INTRODUCTION

Free-swimming mammalian sperm recovered from the site of fertilization in the ampulla of the oviduct typically exhibit a movement pattern known as hyperactivation (review, Yanagimachi, 1981; Cummins, 1982; Suárez et al., 1983). This swimming pattern differs dramatically from that of ejaculated sperm. The flagella of ejaculated sperm propagate three-dimensional beats; as a result, the sperm roll as they swim in linear trajectories (Yeung and Woolley, 1984). The flagella of fully hyperactivated sperm generate asymmetrical, planar beats; as a result, the sperm tend to swim in nonprogressive, circular trajectories (Suárez et al., 1983). Furthermore, the degree of bending of the flagellum in one direction is significantly increased with the onset of hyperactivation (Katz et al., 1978). Hyperactivation has been reported to occur only in sperm removed from the oviduct and not the uterus (Cooper et al., 1979); therefore it has been assumed to be initiated in the oviduct. Based on the assumption that it is initiated in the oviduct, three functions have been suggested for hyperactivated motility: 1.) providing a search pattern for finding the egg (Katz et al., 1978), 2.) providing increased capability for generating thrust against the egg vestments (Katz et al., 1978), and 3.) resisting confinement by folds in the wall of the oviduct (Suárez et al., 1983). Actually, the time and site of the initiation of hyperactivation are not precisely known. Such knowledge would illuminate the functions of this activity and indicate the source of the mechanism triggering its onset. In this study, advantage was taken of the fact that the mouse female reproductive tract is transparent enough to allow observation of sperm within its lumen. Sperm were observed and videotaped within the oviduct and uterus at various times after mating. Measurements were taken from the videotapes and analyzed to determine where and when hyperactivated motility occurs.

The length of mouse sperm, 124 μm (Cummins, 1983), exceeds the diameter of the lumen of the mouse female reproductive tract in the uterotubal junction (utj) and at many points along the oviductal isthmus. As a result, the sperm are constrained enough in their movements that the shape of their swimming trajectory cannot be accurately assessed; therefore, the only measurement that could be

Accepted December 11, 1986.
Received August 18, 1986.
[1] This work was supported by NIH Research Grant HD 19584.
[2] Reprint requests.

taken to detect the onset of hyperactivated motility was of the curvature of the flagellum. To correlate the extent of flagellar bending with the form of the swimming trajectory, the curvatures of the flagella of sperm within the female tract were compared with those of sperm incubated under capacitating conditions in vitro.

MATERIALS AND METHODS

Medium

The medium used for bathing the female reproductive tracts had the following composition: 110 mM NaCl, 2.68 mM KCl, 0.36 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 25.0 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2.4 mM $CaCl_2$, 0.49 mM $MgCl_2$, 5.56 mM glucose, 1.0 mM pyruvate, and 0.006% Na penicillin G, pH 7.6. The medium was sterilized by filtration through a Millex-GV 0.22-μm filter and kept frozen in aliquots. For incubation of the sperm in vitro, 20 mg/ml of Fraction V bovine serum albumin (Sigma A2153; Sigma Chemical Co., St. Louis, MO) were added to the medium. All organics were purchased from Sigma Chemical Co.

Video Equipment

The sperm were videotaped at a rate of 60 fields per second in 1/500-s exposures by a Tritronics shuttered high-speed video camera (Burbank, CA). A record of elapsed time in 0.01-s intervals, generated by a ForA video time generator (model VTG 33, Los Angeles, CA), was simultaneously recorded on a JVC 3/4-inch (2.6 cm) U-matic video cassette recorder (model CR06600U, Victor Co., Japan).

Videotaping Sperm in the Female Reproductive Tract

Adult outbred Swiss mice from Charles River (Kingston, NY) were maintained on a reversed light cycle with the lights turning off at 0930 h and on a 1930 h. Five males were used. Two virgin females were placed with each male at 0930 h and were checked each hour for the presence of copulatory plugs. Presumably, the females that were entering estrus, as they do in the evening, would mate (Rafferty, 1970). The females that mated were killed one hour after the appearance of the copulatory plug (i.e., 1–2 h postcoitus [pc]) or at 1730 or 1930 h. These times represent, respectively, approximately one h before and one h after ovulation, as determined in pilot experiments. Under these conditions, mating generally occurred between 1130 and 1330 h (Suárez, 1986).

At the appropriate time, the mated female was killed by CO_2 inhalation and immediately placed in a 37°C chamber. Each oviduct, as well as the ovary and part of the uterine horn, was removed. The uterine horn was then trimmed down to about 5 mm and the ovary was removed by cutting through the ovarian bursa. The preparation was rinsed quickly with medium, placed on a slide, covered with medium, and capped by a coverslip supported by a 10:1 Vaseline/paraffin mixture. The slide was placed on a microscope stage prewarmed to 37°C by an Arenberg Sage Air Curtain (model 279, Boston, MA). Sperm were located within the oviduct at 100 × magnification; then the magnification was increased fourfold and the movements of the sperm were recorded on videotape through Zeiss differential-interference contrast optics modified by removing the lower polarizing filter. Each slide was videotaped for about 10 min. The flagellar bending of up to 20 sperm that were in focus within each visible part of the tract was analyzed.

Measurements were made of the flagellar curvature ratio (fcr; Suarez et al., 1983) from the frame of videotape in which the flagellum achieved its maximal curvature. Briefly, the fcr is measured as the straight-line distance from the head/midpiece junction to the first geometric inflection point of the tail, divided by the curvilinear distance between these two points measured along the flagellum. In the mouse, an acute but minor bend occasionally forms in the neck and proximal middle piece, a distance of about 10 μm along the tail. This bend is not indicative of flagellar bending as a whole and therefore was not included in the measurements taken to obtain the fcr (see Fig. 1). Lower fcrs are associated with more acute flagellar bending.

Analyzing Sperm Movement In Vitro

The sperm of four of the five males used to mate with the females in the first part of this study were collected and incubated for observation of the onset of hyperactivated motility in vitro. To collect

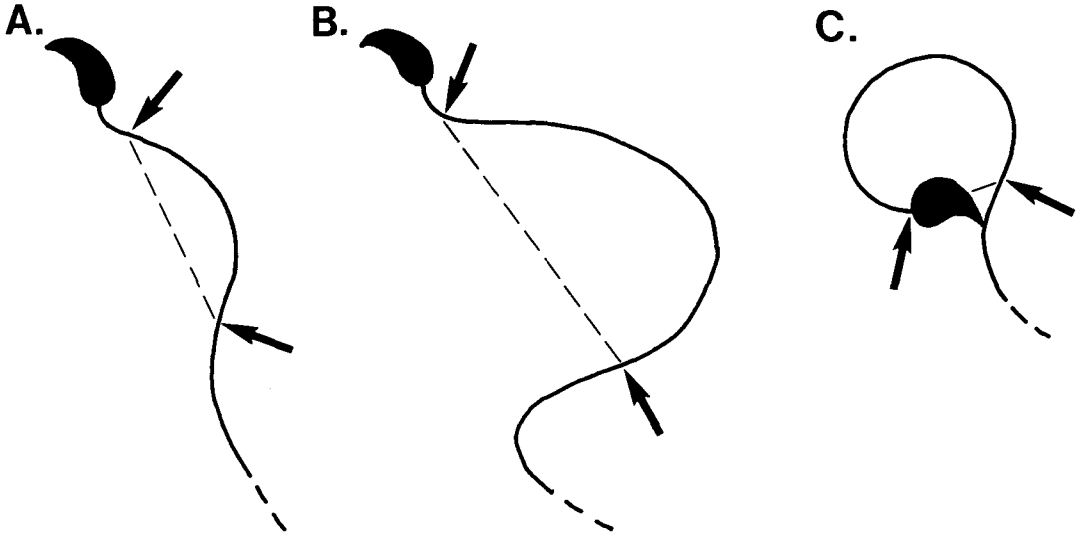

FIG. 1. Measurement of the flagellar curvature ratio in mouse sperm. The *solid lines* depict the portion of the tail that was in focus. The ratio is the straight line distance between the *arrows* (*dashed lines*), divided by the curvilinear distance along the tail between the *arrows* (*solid lines*). If a minor bend appeared behind the head (*A* and *B*), it was not included in the measurement. The point distal to the head is the point of inflection, marking the end of the principal bend. The flagellar curvature ratios of these examples are: A, 0.83; B, 0.58; C, 0.17.

the sperm, the males were killed by CO_2 inhalation and placed inside a 37°C chamber. Their caudae epididymidis were removed, rinsed with medium, and placed together in a 35-mm plastic petri dish (Falcon 1008; Falcon Plastics, Oxnard, CA) containing 3 ml of medium. Each epididymis was held under slight pressure by tweezers and punctured about four times with a 27-gauge needle. The dish was incubated for 10 min to allow motile sperm to disperse. Afterwards, one ml of fluid was carefully withdrawn from the area of the dish farthest from the epididymides. This sample, which contained highly motile sperm, was equally partitioned into four 5-ml plastic tubes (Falcon 2054). Sperm numbers were adjusted to 2×10^6/ml by addition of up to 0.5 ml medium, and the tubes were placed in a 37°C incubator with an atmosphere of 5% CO_2 in air. This method minimized handling of the sperm and maximized their motility.

Fifty-μl samples of sperm were taken for analysis after 0 min, 30 min, and 90 min of incubation. The samples were placed on slides coated with silicone (Sigmacote, Sigma Chemical Co.) and covered by silicone-coated coverslips supported by two strips of Parafilm (American Can Co., Greenwich, CT) to achieve a chamber approximately 100-μm deep. The percentage of motile sperm was determined objectively from the first 100 sperm encountered on randomly chosen microscopic fields of the slide. Then ten fields were videotaped through Zeiss phase-contrast optics. The movement characteristics of the first 10 sperm to swim through the central area of the video screen were analyzed for each time point. The net velocity, average path velocity, and sperm-head velocity (Suárez et al., 1983) were determined for each sperm by measuring the distances traversed in one second. This was approximately the time taken to swim across the video screen field. Briefly, the net velocity is derived from the net distance traveled by the head/midpiece junction (Tessler and Olds-Clarke, 1985), the average path velocity from the distance traveled by the entire cell (taken by measuring the path of the axis about which the head/midpiece junction oscillates), and the sperm-head velocity from the total distance traversed by the head/midpiece junction (Suárez et al., 1983). The fcr was measured at the point that the flagellum of each sperm achieved its maximal bend (see above). A measure of linearity of the trajectory was derived by dividing the net velocity by the average path velocity. This is the formula that defines the "linear index" (Tessler and Olds-Clarke, 1985), but since the average path velocity was obtained differently, we did not use this term.

Statistics

Analysis of variance was used to compare movement characteristics at 0 and 90 min in vitro and to compare the data taken from sperm in the ovi-

duct with those of sperm incubated in vitro. The mixed model of analysis of variance was used, with incubation time or tract site being considered as fixed factors and males as a random factor. Flagellar curvature ratios and the ratios of net velocity/average path velocity must fall between 0 and 1.0, so they had to be normalized by the transformation arc sine$\sqrt{x}$ for analysis of variance (Sokal and Rohlf, 1981). Probabilities of less than 0.05 were considered significant for the rejection of the null hypothesis.

RESULTS

Sperm in the Reproductive Tract

The transport and distribution of mouse sperm in the oviduct, observed in these and similar experiments, have been reported previously (Suárez, 1986). Sperm were not always present in all of the regions of the oviduct, because their distribution was dependent upon the time of observation. Furthermore, since the oviduct is coiled, not all segments were accessible for observation in each preparation. No attempt was made to rearrange the coils, because this was found in preliminary experiments to result in contractions that sometimes redistributed luminal contents. Consequently, the number of sperm sampled varied with each experiment. Nevertheless, sampling was as complete as possible, and did yield information about the motility of sperm within the upper regions of the female reproductive tract.

Table 1 reports the number of sperm sampled in each region at each time point and the mean fcrs of the sampled sperm. The lower isthmus was the only area in which data were obtained for all time points and males. In this region, the fcrs were significantly lower at 1730 h than at the other time points ($p<0.05$). Ovulation occurred between 1730 and 1930 h in all females except those housed with Male A; in this case, eggs were seen in the ampulla at the earlier time. Because the female mice were not preovulatory, the data from the Male A at 1730 h were not included in the analysis.

As reported previously (Suárez, 1986), the sperm in the uterus, uterotubal junction, and isthmus appeared to adhere to the epithelium most of the time, only breaking away occasionally. The movement of both free-swimming and attached uterine

TABLE 1. The flagellar curvature ratios for mouse sperm videotaped in the female tract.

Site	Male	1–2 h postcoitus	1730 h (preovulatory)	1930 h (postovulatory)
Colliculus tubarius	A		0.84 ± 0.017(3)*	
	B	$0.78 \pm 0.020(10)^{90}$	0.69 ± 0.045(3)	
	C	$0.80 \pm 0.024(8)^{90}$	0.83 ± 0.008(2)	
	D	0.62 ± 0.012(2)		
Intramural junction	C		0.81 ± 0.027(6)	
	E	0.80 ± 0.030(6)		
Extramural junction	A	0.76 ± 0.048(2)	$0.77 \pm 0.025(9)^{90}$	
	B		0.77 ± 0.037(4)	
	C	$0.70 \pm 0.035(9)^{90}$	$0.78 \pm 0.030(5)^{90}$	0.68 ± 0.012(7)
	D	$0.70 \pm 0.024(10)^{90}$	0.70 ± 0.060(3)	
	E		0.77 ± 0.022(11)	0.71 ± 0.012(5)
Lower isthmus	A	$0.73 \pm 0.036(8)^{90}$	0.74 ± 0.012(8)**	$0.76 \pm 0.027(17)^{90}$
	B	$0.73 \pm 0.035(9)^{90}$	$0.87 \pm 0.014(12)^{0}$	$0.77 \pm 0.016(19)^{90}$
	C	$0.78 \pm 0.011(5)^{90}$	$0.83 \pm 0.024(7)^{0}$	$0.64 \pm 0.025(10)^{90}$
	D	0.84 ± 0.004(2)	$0.80 \pm 0.028(6)^{0}$	$0.70 \pm 0.022(14)^{90}$
	E	0.82 ± 0.034(9)	0.80 ± 0.020(6)	0.63 ± 0.047(8)
Upper isthmus	A			0.72 ± 0.012(3)
	D		0.82 ± 0.035(5)	
	E		0.80 ± 0.018(3)	0.59 ± 0.063(3)
Ampulla	D		0.68 ± 0.100(2)	0.62 ± 0.067(5)

*Mean flagellar curvature ratio ± SEM. The numbers in parentheses are the number of sperm.

**Ovulation had occurred at this time point in this female, so the data were not included in the statistical analyses.

^{0,90}Analysis of variance found no overall difference ($p>0.05$) to exist between sperm of the indicated males at these times and places in vivo and the same males' sperm incubated for $0(^{0})$ or $90(^{90})$ min in vitro. Conversely, the measurements of in vivo sperm were significantly different from the other in vitro time point.

sperm was sinuous, appearing as though the sperm were swimming in a medium more viscous than the luminal fluid of the oviduct. Oviductal sperm appeared more active. When they were not attached to the epithelium, they often appeared to be hyperactivated, because their motility was vigorous and not linear. The few ampullar sperm observed were exceptionally active, so that not all could be tracked for videotaping. Nevertheless, one could not judge them to be more active than isthmic sperm, because they were less constrained by the limits of the lumen and the presence of numerous other sperm than were the isthmic sperm.

At each time point, as reported previously (Suárez, 1986), a segment of the lower isthmus of some females contained immotile sperm. Measurements could not be taken of these sperm, because they were too densely packed in the lumen.

Sperm Incubated In Vitro

The percentage of motile sperm remained high throughout the incubation period. At the beginning of the incubation period, the mean percentage of motile sperm for all males was 75 (range 70–80). At 30 min, the mean was 76 (70–80), and at 90 min it was 69 (65–80).

Figure 2 depicts the means of the maximal fcrs. These values differed significantly between the males, yet all were significantly lower after 90 min of incubation, signifying that bending became more acute with time. The flagellar bending was highly asymmetrical, with the tail bending more acutely in the opposite direction to the curve of the head. In other words, the tip of the head pointed outward during the acute bends.

The sperm head and average path velocities were significantly greater at 90 min than at the beginning of the incubation (Figs. 3 and 4). The average path velocity/net velocity was significantly lower at 90 min, indicating that the trajectories became less linear (Fig. 5) and therefore more characteristic of hyperactivation with time. There was no significant variation attributable to males for any of these parameters.

Comparison of Sperm Incubated In Vitro with Sperm in the Tract

The fcrs of sperm incubated for 0 and 90 min in vitro were compared by analysis of variance with those of sperm in the female tract when the

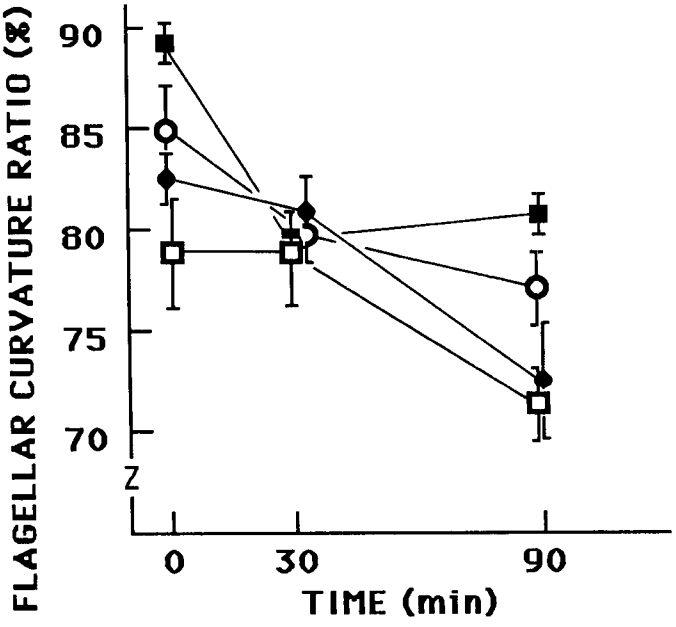

FIG. 2. The mean maximum flagellar curvature ratios of sperm incubated in vitro. The sperm of each male are represented by the symbols: (●) Male A, (○) Male B, (■) Male C, and (□) Male D. The *bars* represent the standard errors of the mean. Decreasing values represent increasingly acute flagellar bends.

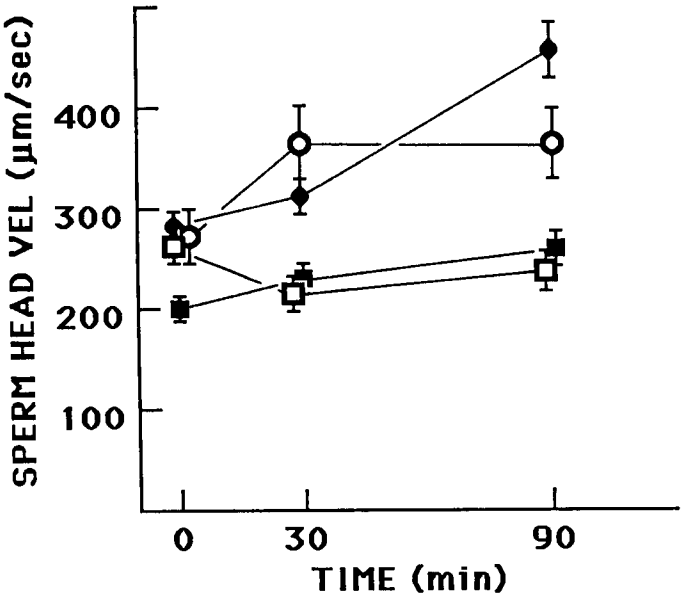

FIG. 3. The mean sperm head velocities (*SPERM HEAD VEL*) of sperm incubated in vitro. The sperm of each male are represented by the symbols: (●) Male A, (○) Male B, (■) Male C, and (□) Male D. The *bars* represent the standard errors of the means of ten sperm.

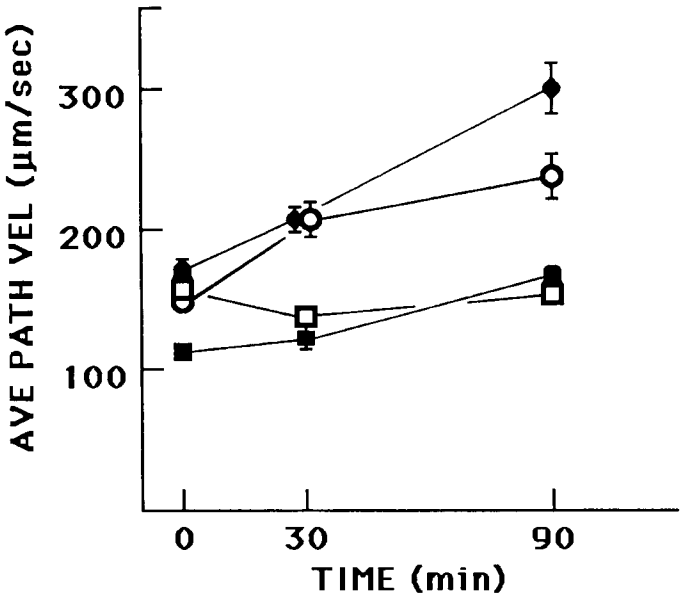

FIG. 4. The mean average path velocities (*AVE PATH VEL*) of sperm incubated in vitro. The sperm of each male are represented by the symbols: (●) Male A, (○) Male B, (■) Male C, and (□) Male D. The *bars* represent the standard errors of the means of ten sperm.

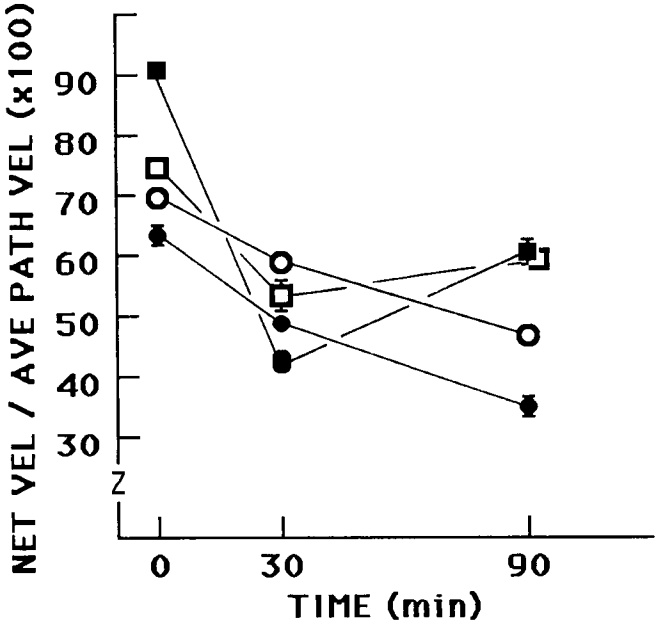

FIG. 5. The mean net velocity (*NET VEL*)/average path velocity (*AVE PATH VEL*) of sperm incubated in vitro. The sperm of each male are represented by the symbols: (●) Male A, (○) Male B, (■) Male C, and (□) Male D. The *bars* represent the standard errors of the means of ten sperm. Deceasing values represent decreasing linearity of trajectory.

number of tract sperm that could be measured in each sample was not less than five and sufficient data were available for at least two males (see Table 1). For example, 10 and 8 sperm could be measured for Males B and C, respectively, at the colliculus tubarius at 1–2 h pc, so these data were compared with those of sperm from the same males that were incubated in vitro. Sperm measured at the colliculus tubarius and in the extramural utj (Males C, D) and lower isthmus (Males A, B, C) at 1–2 h postcoitus had significantly different fcrs from those incubated for 0 min in vitro, but not from those of sperm incubated for 90 min in vitro (Table 1). This indicates that the sperm were executing the same acute flagellar bends in vivo as sperm samples that had begun to assume nonlinear movement in vitro—movement that is characteristic of hyperactivated motility. This was also true of sperm in the extramural utj at 1730 h (Males A, C) and the lower isthmus at 1930 h (Males A, B, C, D). Only sperm sampled from the lower isthmus at 1730 h had fcrs that did not differ from sperm incubated for 0 min in vitro (Males B, C, D). These sperm, as a whole, were not exhibiting the increased flagellar bending observed after 90 min in vitro and associated with the onset of hyperactivated motility.

There were insufficient numbers of measurable sperm recorded in the upper isthmus and ampulla for any statistical analysis. Since these segments were relatively transparent and amenable to observation, there actually were very few sperm present.

DISCUSSION

We found the change in motility of mouse sperm incubated in vitro to be gradual. During the course of the incubation, sperm began to make intermittent acute flagellar bends that caused them to change direction. With time, the bends occurred more frequently, until some sperm swam in circles. Superimposed on this change was an increase in the yaw of the head during progressive, linear displacement, which made the pattern of swimming appear helical. These changes were difficult to assess qualitatively, but the measurement of maximal fcrs and linear indices made it possible to determine quantitatively when a statistically significant change had occurred in the population. Thus we were able to say that at 90 min the fcr and swimming progressiveness were significantly lowered. Hyperactivated motility has been defined as being

distinguished from the motility of sperm in the ejaculate by significantly lower fcrs and progressiveness ratios (Suarez et al., 1983); by this definition, hyperactivation had been initiated in these mouse sperm by 90 min of incubation in vitro. We stress that "initiated" means that hyperactivated flagellar bending has occurred, even though it may have been intermittent in nature. In this study, spatial limitations imposed upon sperm in the oviduct prohibited measurement of velocity parameters that would be used to quantitate progressiveness. To determine whether sperm in the oviduct had initiated hyperactivation, their fcrs had to be compared with those of sperm incubated under capacitating conditions in vitro that also had significantly lowered linearity. Thus the sperm in vitro provided a scale against which to assess the flagellar bending of the sperm in vivo.

Other investigators have reported similar observations of mouse sperm motility in vitro. Olds-Clarke (1985) correlated a decrease in linearity of trajectory during incubation in vitro with capacitation and in vitro fertilization. Fraser, who was the first to describe hyperactivation in the mouse (Fraser, 1977), has used a 0—+++ subjective system (Fraser, 1983, 1984) or a general estimate (Fraser and Quinn, 1981; Fraser, 1985) to report hyperactivation in vitro and to relate it to fertilizing ability. What Fraser was calling hyperactivation (or +++) is apparently identical behavior to what we observed in vitro (Fraser, personal communication). Cooper (1984) classified mouse sperm as hyperactivated if they exhibited large-amplitude flagellar bends and lateral head displacements.

In this study, hyperactivated flagellar bending occurred 1—2 h pc in the uterus as well as the isthmus. Olds-Clarke (1986) found that sperm "stripped' from the isthmus into medium were less progressive than sperm stripped from the uterus at one h pc. Her data imply that hyperactivation is not initiated or is not as advanced in the uterus as in the isthmus at one h pc. Our sperm were measured in situ without dilution and were in a selected portion of the uterus near the entrance to the isthmus. In addition, we found that many of the sperm observed in situ were attached to the epithelium by their heads (Suárez, 1986). Her stripping technique may have recovered fewer attached sperm than free-swimming sperm, thereby sampling a different portion of the uterine and isthmic populations.

Gaddum-Rosse (1981) observed that straight-swimming rat sperm passed through the utj from the uterus, while immature sperm that swam in circles did not. The failure of the latter to traverse the utj, however, may have been the fault of their immaturity, rather than their circular motility. Nevertheless, we do not know whether the acutely bending sperm we observed at the entrance to the utj would have reached the oviduct.

At only one time and site in vivo did we find the fcrs to be similar to those of sperm analyzed at 0 min in vitro. This was in the lower isthmus at 1730 h, i.e., approximately 1—2 h before ovulation. This time point is when the greatest occurrence of immotile sperm in the lower isthmus was noted (Suarez, 1986). The unknown factor responsible for the complete suppression of motility could also have suppressed the bending of sperm that were not completely immobilized. This may have been terminated by the occurrence of ovulation, resulting in the more acute bending found approximately 1—2 h after the time of ovulation. Suppression of motility in the lower isthmus has also been reported for the rabbit (Cooper et al., 1979; Overstreet et al., 1980).

The parameters used to quantify sperm motility have varied considerably. Some of this variation is attributable to species differences. For example, we found it impossible to measure roll and beat frequencies in mouse sperm by using the same video equipment as was used to measure these parameters in the rabbit (Suárez et al., 1983) and hamster (Suárez et al., 1984). The equipment used has also affected the measurement parameter selected or the resulting data. For example, the sperm-head velocity, also referred to as the curvilinear velocity (Tessler and Olds-Clarke, 1985), has been found to be dependent upon the framing or fielding frequencies of the recording equipment (Katz and Phillips, 1986). This is because determining sperm-head velocity involves measuring the path of the head, which moves from side to side as the entire sperm cell moves. Slower recording frequencies capture less of the lateral movement of the head. We compared our mean values of sperm-head velocity for all sperm at 0 and 90 min of incubation, and found

that at 60 fields/s, they were 265 and 324 μm/s, respectively, while at 30 fields/s (using every other field), the velocities of the same sperm were only 189 and 244 μm/s. The data taken at 30 fields/s were closer to those reported by Tessler and Olds-clarke (1985), whose equipment recorded at the slower frequency. The average path velocity, as we measure it, is affected very little by framing frequency, because it measures the translation of the entire cell and omits the lateral movement of the head. We therefore propose that average path velocity may be more useful for comparing data from different laboratories. Average path velocity has also been measured as a five-point moving average of the digitized sperm head track (Tessler and Olds-Clarke, 1985; Olds-Clarke, 1986). We were unable to compare our values with these (Tessler and Olds-Clarke, 1985), because the latter were not reported directly, but rather were divided by net velocities to compute linearity of trajectory.

The occurrence of acute bending at a time long before ovulation and a location distal to the site of fertilization indicates that hyperactivation serves functions additional to searching for the egg mass and penetrating the egg vestments, as originally proposed by Katz et al. (1978). We observed mouse sperm confined in pockets of the lower isthmus to bend sharply, turn toward the central lumen, and then escape. Our observations provide new evidence for the more recently proposed hypothesis that hyperactivated sperm resist confinement by mucosal folds better than pre-hyperactivated sperm (Suárez et al., 1983). This was originally proposed for ampullar sperm, but could apply to sperm in the isthmus and utj as well, especially in species having complex mucosal folds in those regions (see Nalbandov, 1969).

ACKNOWLEDGMENTS

We would like to thank Dr. Irwin Liu for allowing us to use his equipment and space. We would also like to thank Wayne Gottlieb for his computer-drawn graphics and Dr. Stanley Meizel for his suggestions regarding the manuscript.

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