

The Effects of Cyclic Adenine Nucleotides, Phosphodiesterase Inhibitors, and Cauda Epididymidal Fluid on the Motility of Rat Epididymal Spermatozoa

T. T. TURNER AND R. D. GILES

*From the Department of Urology,
University of Virginia School of Medicine,
Charlottesville, Virginia*

Experiments were performed to determine whether the rat epididymis secretes a "forward-motility" factor(s) similar to that found by others in the bovine epididymis. Lumen content of the rat caput and cauda epididymidis was collected by micropuncture. Caput and cauda spermatozoa were diluted in .154 M NaCl, 25 mM theophylline, or 10 mM cAMP, dibutyryl cAMP, 8-bromo cAMP, or 8-bromo AMP in saline. Progressive motility was judged by determining linear distance traveled by sperm in the various diluents after 30-minute incubation at 37 C. Neither theophylline nor cyclic adenine nucleotides cause caput sperm to swim distances attained by cauda spermatozoa. In other experiments, caput spermatozoa were preincubated for either 5 or 30 minutes at either 32 C or 37 C with fresh cauda lumen fluid prior to dilution with the test solutions. Cauda fluid did not significantly enhance the progressive motility of caput sperm in any diluent. Stimulation with 25 mM theophylline always resulted in more distance traveled than with any other diluent. This effect was reproduced by 25 mM caffeine, another xanthine phosphodiesterase inhibitor (PDI). There was no stimulation of motility with 200 μ M papaverine, a nonxanthine PDI. The results of this study failed to demonstrate the presence of a factor in lumen fluid of the distal rat epididymis that acts in conjunction with cyclic adenine nucleotides to induce mature cell motility in immature spermatozoa.

Key words: spermatozoa, motility, epididymal, rat.

Mammalian sperm develop the capacity for motility during their transport through the epididymis. Previous investigations using a variety of mammalian species have presented evidence that a range of substances, including Ca^{++} (Morton et al., 1978; Morton et al, 1979), cyclic adenosine 3':5' monophosphate (cAMP; Frenkel et

al 1973; Hoskins et al, 1975; Morton et al, 1978; Brandt and Hoskins, 1980), epididymal "forward motility protein" (FMP, Hoskins et al, 1978; Brandt et al, 1978), neurotransmitters (see Nelson et al, 1980), and guanosine triphosphate and reproductive tract polyamines (Casillas et al, 1980), are all involved in the development or initiation of mammalian sperm motility. It is difficult to synthesize an overview of the biochemical sequence leading to progressive sperm motility since it is entirely possible that processes important in one species may be totally irrelevant in another. Nevertheless, an assimilation of loose-knit pieces of information into a generalized concept can be stated in the following manner. Neurotransmitters increase Ca^{++} transport through sperm membrane ionophoric channels; Ca^{++} , perhaps along with guanine nucleotides and spermine, serves as a modulator of adenylate cyclase activity which in turn catalyzes intracellular cAMP synthesis. Increased intracellular cAMP, in conjunction with an extracellular, possibly cell-membrane-bound FMP, allows or stimulates a cascade of intracellular molecular events leading to sperm motility. Recently, Brandt and Hoskins (1980) reported that increased intracellular cAMP concentrations stimulate cAMP-dependent protein kinase which presumably phosphorylates intracellular sperm motility protein.

This scheme is perhaps partly accurate for some species and entirely accurate for none. Previous reports from this laboratory, for example, have demonstrated that rat sperm initiate motility in a Ca^{++} -free medium (Turner and Howards, 1978),

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Reprint requests: Terry T. Turner, Ph.D., Department of Urology, Box 422, University of Virginia School of Medicine, Charlottesville, Virginia 22908.

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and Hoskins et al (1978) have noted that Ca^{++} is not a modulator of bull sperm motility. The present investigation was performed to determine whether the rat epididymis secretes a factor similar to the FMP found in bulls which, when supported by increased cAMP concentrations, will induce progressive motility in immature epididymal spermatozoa.

Materials and Methods

Adult male Sprague-Dawley rats (450–550 g) were obtained from University vivarium sources and were acclimated for at least one week prior to use. Animals were anesthetized with an intraperitoneal injection of urethane solution (.25 mg/ml) at a dose of 1 mg/kg body weight. Micropuncture of the caput and cauda epididymal duct was performed *in vivo* as described by Turner (1979). Immediately after collection of each micropuncture sample, a sperm motility assay was performed. This method (Fig. 1) is a modification of the qualitative microassay for motility described previously (Turner and Howards, 1978) and allows for quantitation of progressive motility.

The "Distance-Traveled" Assay

Microscope culture slide wells were filled with water-equilibrated mineral oil and were warmed to 37 C on a slide-warming tray (Fig. 1a), One-hundred-nl or 500-nl aliquots of micropuncture samples (sperm + fluid) were collected in precalibrated constriction micropipettes and were expressed under the oil in the microscope slide wells (Fig. 1b). Samples were diluted 1:10 with a selected diluent (Fig. 1c) and immediately were scored for motility. A subjective score of 4 indicated maximum motility. Scores were assigned in whole number units down to 0 which indicated absence of motility (see Turner et al, 1978). All samples were imotile prior to dilution. After each sample was scored, a prefilled .05 mm × .5 mm × 50 mm glass micropipette (Vitro Dynamics, Rockaway, New Jersey) was inserted into the drop (Fig. 1d). The rectangular micropipette was filled with the same diluent previously applied to the sample. After insertion into the already diluted epididymal sperm sample, the pipette was rested on a small cork support block at an approximate 20° angle. The entire apparatus was covered by a black box and incubated for 30 minutes at 37 C. At the conclusion of the incubation period, the pipette was examined under the light microscope. The forward-most spermatozoan was located; then a mark was made on the pipette surface over the location of the 25th cell. This simple method added a constancy to the assay and was useful in locating the "front" of the mass of cells moving up the lumen of the micropipette. The distance (mm) from the proximal end of the micropipette to the mark on the pipette surface was determined and recorded as the distance traveled by caput or cauda epididymal spermatozoa in that particular diluent (Fig. 1e). The original sample drop was rescored subjectively for motility at 30,

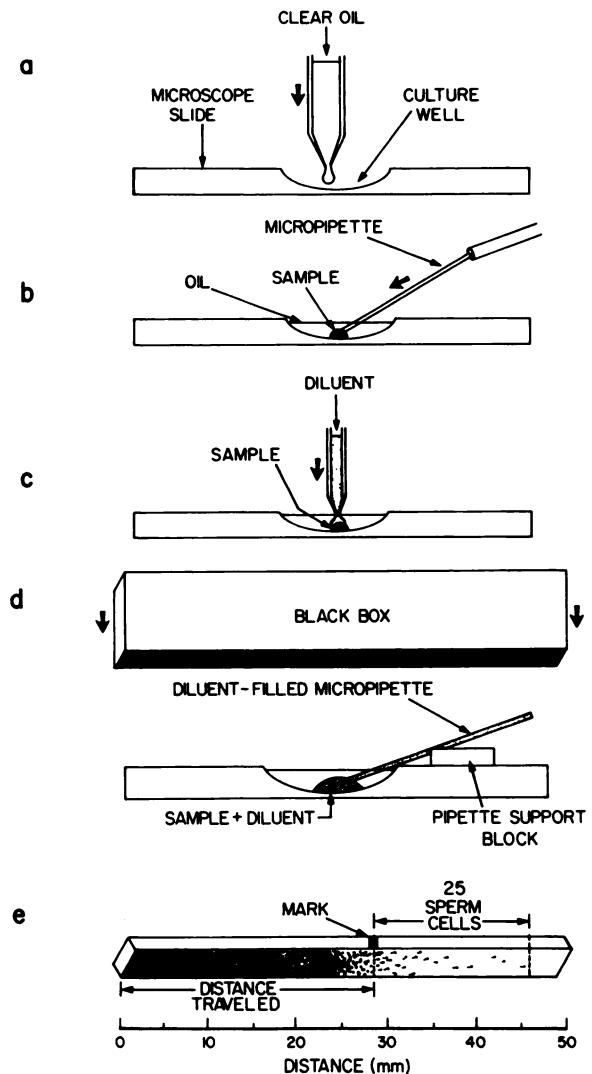


Fig. 1. Schematic representation of the progressive motility assay. The assay allowed subjective scoring of sperm motility plus objective measurement of distance traveled by sperm in different diluents. See text for detailed description of each step.

45, and 60 minutes after beginning the incubation; thus every sample had assigned to it four different subjective motility scores, along with the measurement of the distance traveled during the first 30 minutes of the incubation.

This assay allowed for detection of sperm samples with the appearance of motility but, in fact, little capacity for progressive motility. This was particularly a problem with caput spermatozoa when early experiments revealed the potential for moderate-to-high motility scores but did not indicate an equally high capacity for progressive movement. There is a potential lack of discrimination between samples of short-term but high-grade motility and long-term but low-grade motility. As a practical matter, this did not become a problem in this study.

Diluents

In all experiments, .154 M NaCl was used as control diluent. One, 2, 5, and 25 mM theophylline (T), 25 mM caffeine (CAF), 10 mM cAMP, 10 mM dibutyl cyclic adenosine 3':5' monophosphate (dBcAMP), 10 mM 8-bromocyclic adenosine 3':5' monophosphate (8BcAMP), 10 mM 8-bromo adenosine monophosphate (8BAMP), 200 μ M papaverine (PAP), and 10 mM SQ20006 were constituted in saline and adjusted to a final pH of 6.8–7.2. Cell-free cauda epididymidal fluid was obtained by centrifugation of cauda epididymidal micropuncture samples for 15 minutes at 3,600 g, 0 C. The sperm pellet was discarded, and the cauda epididymidal fluid was used as a diluent when required.

Experiments

Caput and cauda spermatozoa obtained by *in vivo* micropuncture were diluted with .154 M NaCl, 25 mM T, 10 mM cAMP, 10 mM dBcAMP, 10 mM 8BcAMP, or 10 mM 8BAMP. The diluents were also used on 100-nl caput samples which had been incubated previously with 1 μ l cell-free cauda fluid. In early experiments, cauda fluid was incubated with caput sperm cells at either 32 or 37 C for either 5 or 30 minutes. Changing these parameters did not affect the results achieved; therefore, the procedure was standardized to a 5-minute incubation at 32 C. The motility scores and distance traveled by spermatozoa in each diluent were recorded and analyzed statistically.

A second series of experiments used .154 M NaCl; 1, 2, and 5 mM T; 2 mM T + 10 mM cAMP; and 25 mM CAF. The nonxanthine phosphodiesterase inhibitors (PDI), PAP and SQ20006, were used at concentrations of 200 μ M and 10 mM, respectively.

Data Analysis

Within each diluent, distance traveled by caput sperm alone and caput sperm + cauda fluid were compared with distance traveled by cauda sperm (their control) by the Student's *t* test. Diluent effects on each type of spermatozoa were determined by analysis of variance and the Duncan's multiple range test.

Results

Spermatozoa in micropuncture samples obtained from the lumen of the cauda epididymidis exhibited maximum or near-maximum initial motility scores in all diluents tested (Table 1). However, 30-minute motility scores and the distance traveled by the sperm in the various diluents after a 30-minute incubation varied considerably (Table 1). Data from the motility assay, representing the distance traveled, are illustrated in Fig. 2, which also groups the data according to diluent used and provides statistical analysis. Cauda spermatozoa diluted in .154 M NaCl traveled approximately 20 mm in the micropipette. Stimulation with 25 mM T resulted in a significantly greater distance travelled (approximately 40 mm; $P < 0.05$). In contrast, addition of 10 mM dBcAMP reduced the distance traveled to only 12 mm, a distance significantly smaller ($P < 0.05$) than that traveled by saline-diluted cauda sperm ($P < 0.05$).

Caput spermatozoa in the various diluents had

TABLE 1. The Effect of Theophylline and Some Adenine Nucleotides on the Motility of Cells from the Caput and Cauda Epididymidis

Spermatozoa	Diluent	No. of Samples Observed	Mean \pm SE Motility Score				
			0	30 min	DT-30 min*	45 min	60 min
Cauda cells in cauda fluid	.154 M NaCl	24†	4.0 \pm 0.0	2.3 \pm 0.1	19.6 \pm 1.3	1.2 \pm 0.2	0.6 \pm 0.2
	25 mM T	15	4.0 \pm 0.0	1.9 \pm 0.1	41.3 \pm 1.3	1.0 \pm 0.2	0.4 \pm 0.2
	10 mM cAMP	11	3.9 \pm 0.1	2.4 \pm 0.2	16.7 \pm 1.0	1.4 \pm 0.2	0.6 \pm 0.2
	10 mM dBcAMP	8	3.9 \pm 0.1	0.7 \pm 0.3	12.1 \pm 1.1	0.1 \pm 0.1	0.1 \pm 0.1
	10 mM 8BcAMP	5	4.0 \pm 0.0	1.4 \pm 0.5	20.4 \pm 5.7	0.0	0.0
	10 mM 8BAMP	10	3.8 \pm 0.2	1.8 \pm 0.4	12.6 \pm 1.2	0.4 \pm 0.2	0.0
Caput cells in caput fluid	.154 M NaCl	18	0.6 \pm 0.2	0.0	12.3 \pm 1.1	0.0	0.0
	25 mM T	7	2.6 \pm 0.3	0.7 \pm 0.4	33.1 \pm 3.1	0.4 \pm 0.3	0.1 \pm 0.1
	10 mM cAMP	9	0.9 \pm 0.1	0.0	9.1 \pm 1.4	0.0	0.0
	10 mM dBcAMP	7	0.6 \pm 0.2	1.1 \pm 0.3	10.0 \pm 0.8	0.4 \pm 0.2	0.0
	10 mM 8BcAMP	7	3.0 \pm 0.4	1.4 \pm 0.3	10.0 \pm 1.0	0.4 \pm 0.2	0.0
	10 mM 8BAMP	5	1.2 \pm 0.4	1.2 \pm 0.6	7.5 \pm 0.9	1.0 \pm 0.6	0.6 \pm 0.4
Caput cells in cauda fluid	.154 M NaCl	11	0.2 \pm 0.1	1.0 \pm 0.1	14.4 \pm 1.1	0.8 \pm 0.2	0.7 \pm 0.1
	25 mM T	14	1.9 \pm 0.1	2.2 \pm 0.3	30.0 \pm 2.8	2.1 \pm 0.2	1.9 \pm 0.3
	10 mM cAMP	11	0.1 \pm 0.1	0.8 \pm 0.1	11.2 \pm 0.4	0.8 \pm 0.1	0.7 \pm 0.1
	10 mM dBcAMP	11	0.3 \pm 0.1	2.4 \pm 0.3	9.7 \pm 1.0	2.2 \pm 0.2	1.6 \pm 0.3
	10 mM 8BcAMP	5	1.4 \pm 0.4	2.2 \pm 0.4	13.4 \pm 0.7	1.8 \pm 0.2	1.8 \pm 0.2
	10 mM 8BAMP	6	1.2 \pm 0.3	1.8 \pm 0.2	9.1 \pm 0.5	2.0 \pm 0.0	2.0 \pm 0.0

* Distance traveled (mm) up micropipette after 30 minutes.

† All animals had cauda cells diluted in control diluent to insure that mature-cell motility was normal.

very low initial motility scores, except for the scores of $2.6 \pm .03$ and $3.0 \pm .04$ in 25 mM T and 10 mM 8BcAMP, respectively (Table 1). Linear distance traveled by control caput sperm (in saline diluent) was approximately 12 mm, a distance significantly less than that traveled by control cauda sperm ($P < 0.01$, Fig. 2). Caput sperm traveled significantly smaller distances than cauda cells in the same diluents, except for dBcAMP and 8BAMP (Fig. 2). Addition of 25 mM T resulted in a significantly greater ($P < 0.05$) distance traveled by caput cells than did addition of any other diluent. This distance was still significantly less ($P < 0.01$) than that traveled by cauda cells in 25 mM T (Fig. 2).

Caput cells preincubated in cauda fluid evidenced low initial motility scores; however, after 30 minutes, the scores usually appeared to be enhanced in the presence of the cauda fluid (Table 1). Caput cells in cauda fluid moved approximately 14 mm in the micropipettes 30 minutes after dilution with .154 NaCl. All other diluents gave similar results except 25 mM T which significantly increased ($P < 0.05$) the distance traveled to approximately 30 mm. The distance traveled by preincubated

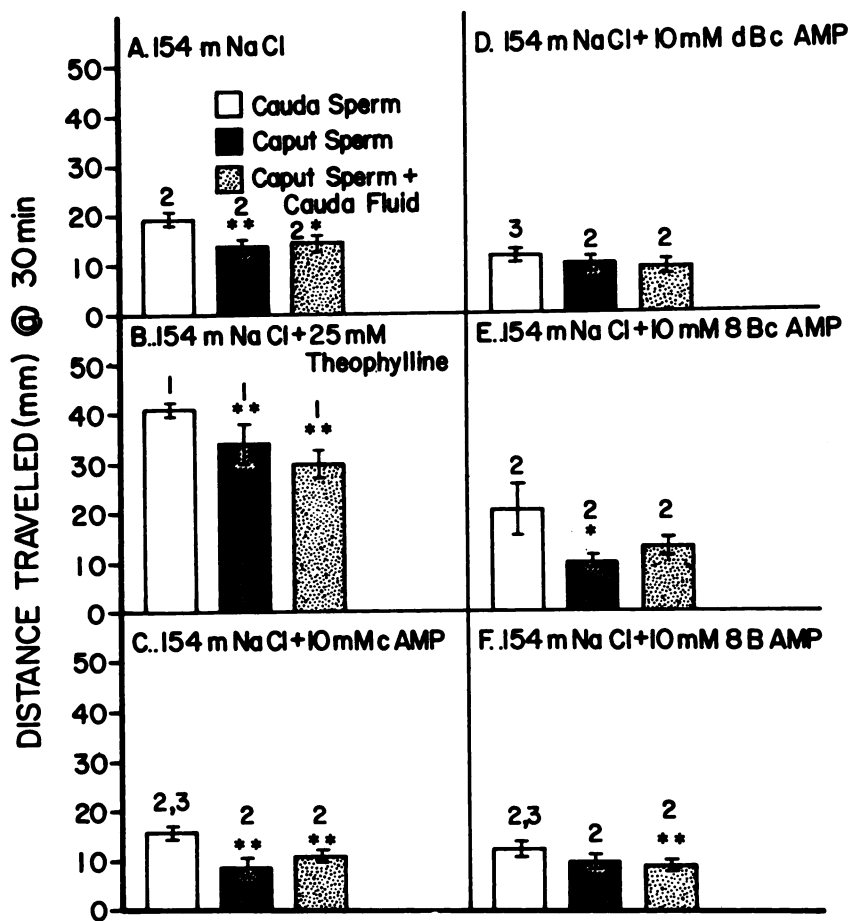
caput sperm in 25 mM T was significantly less ($P < 0.01$) than that traveled by cauda cells in the same diluent (Fig. 2).

In the second series of experiments, both 2 and 5 mM T stimulated caput spermatozoa to swim significantly greater ($P < 0.01$) distances than did the cells diluted in the saline control diluent (Fig. 3). Caput sperm cells diluted with 2 mM T + 10 mM cAMP did not swim a significantly greater distance than did the control caput cells. Dilution of caput sperm with 25 mM CAF and 25 mM T resulted in similar distances traveled (30–35 mm) at the end of a 30-minute incubation. Dilution of caput sperm with 200 μ M PAP provided no stimulation to either motility scores (not shown) or distance traveled (Fig. 3). Motility assays with SQ20006 were not successful due to its insolubility in the basic diluent.

Discussion

Results of the present study do not support the hypothesis that the rat epididymis secretes a factor that would allow caput epididymidal spermatozoa

Fig. 2. Distance traveled by cauda sperm, caput sperm, and caput sperm preincubated in cauda fluid. Distances were measured 30 minutes after addition of the specific diluents. Within each of the three types of spermatozoa, different numbers (1, 2, 3) over each bar indicate that those sperm traveled significantly different ($P < 0.05$) distances in that diluent than did the same type of sperm in the other diluents. Within each of the six diluents, asterisks over the bars indicate that caput sperm or caput sperm in cauda fluid swam significantly a smaller distance in that diluent than did mature cauda spermatozoa (* = $P < 0.05$; ** = $P < 0.01$).



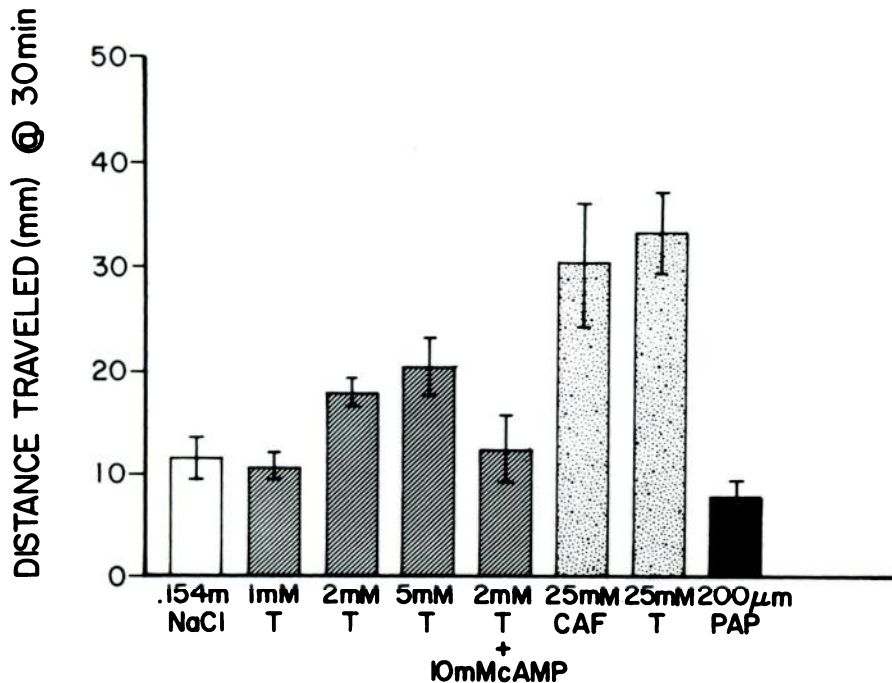


Fig. 3. Distance traveled by caput spermatozoa in different concentrations of xanthine PDI, PDI + cAMP, and a nonxanthine PDI. T and CAF (xanthines) stimulated vigorous flagellation; PAP (nonxanthine) did not.

to exhibit the progressive motility as seen in mature, cauda spermatozoa. If the rat epididymis secretes a factor similar to that found in the bull (Hoskins et al, 1978; Brandt et al, 1978), then cauda epididymidal fluid should contain the factor. Caput cells incubated in cauda epididymidal fluid prior to being presented with increased concentrations of cAMP would have exhibited normal or near-normal progressive motility. This did not happen. Neither cAMP nor its usually more membrane-permeant analogs dBcAMP and 8BcAMP caused caput cells in cauda fluid to exhibit the motility scores or linear distance traveled of mature cauda sperm cells diluted in saline.

Twenty-five mM T caused significant increases in the distance traveled when compared with the other diluents; however, immature sperm diluted in 25 mM T never progressed as far as mature cells diluted in the same diluent (Fig. 2). Caput cells, diluted in 25 mM T, did travel further in the micropipette than did mature cauda cells in saline. This was due to hyperflagellation or violent twitching induced in some caput cells. While not a mature motility pattern, still this caused movement through the fluid and sufficient "rebound" from the pipette walls to produce considerable progression through the pipette. This progression occurred in the absence of the mature and purposeful forward motility pattern seen in cauda spermatozoa. The normal mature-cell motility pattern was not induced by preincubating caput

sperm in cauda epididymidal fluid prior to dilution with T or any of the adenine nucleotides.

Caput spermatozoa incubated in caput fluid and then diluted in 8BcAMP exhibited motility scores similar to those obtained from the same type of cells diluted in 25 mM T (Table 1). However, the distance traveled by 8BcAMP-treated cells was significantly less than that traveled by sperm diluted in 25 mM T (Fig. 2). Addition of 8BcAMP stimulated a large proportion of sperm to exhibit a moderate-frequency, undulating type of motility which obviously was not very efficient in propelling cells forward. The same is true for other cases where immature sperm cells received moderate-to-high motility scores but did not have a similar capacity for actual progressive motility. The qualitative motility scores were a method of recording a subjective impression of motility, an impression which could be influenced by such things as flagellar beat frequency, proportion of cells flagellating, proportion of cells exhibiting progressive motility, or speed of progression. The assay for distance traveled demonstrated that immature spermatozoa can give an impression of motility without having the capacity of mature cells for forward motility.

Of all the diluents tested, the phosphodiesterase inhibitor T was the only one that produced any stimulation of motility. However, this effect consisted of sperm hyper-flagellation without stimulation of normal progressive motility. The experi-

ments reflected in Fig. 3 were performed to determine whether or not phosphodiesterase in the caput lumen fluid was hydrolyzing the 3':5' bonds of cAMP and its analogs, potentially rendering them ineffective as cyclic nucleotide stimulators of motility. Two mM T (see Fig. 3) was determined to be the baseline T concentration necessary for inhibition of lumen phosphodiesterase activity. This low concentration of T caused some stimulation of motility and thus appeared to be sufficient to inhibit the majority of phosphodiesterase present. Any cAMP added to caput samples treated in this manner would presumably be safe from hydrolysis and would remain in the cyclic form of the nucleotide. Figure 3 demonstrates that 2 mM T + 10 mM cAMP did not cause a stimulation of motility. Thus, hydrolysis of the cyclic structure does not appear to be a reason for the noneffectiveness of the cyclic adenine nucleotides.

Figure 3 also demonstrates that another xanthine PDI, caffeine, stimulated caput cell motility above that seen in caput cells diluted in saline. This finding is consonant with the results from other studies regarding caffeine stimulation of caput sperm percent motility (Frenkel et al, 1973; Wyker and Howards, 1977). The nonxanthine PDI, papaverine, in concentrations twice that found to stimulate bull caput sperm tail flagellation (Hoskins et al, 1975), did not stimulate rat caput sperm motility. An explanation for this could be that xanthine PDIs have specific effects on spermatozoa besides their inhibition of phosphodiesterase. This has been reported previously for bulls (Garbers et al, 1973b), boars (Garbers et al, 1973a), and humans (Levin et al, 1981) and is suggested by the present study in that nonxanthine PDI failed to affect caput sperm motility.

While the authors do not challenge the fact that cAMP is required for sperm tail flagellation, they wish to point out the possibility that some effect of xanthine PDIs, which is unrelated to cAMP, may also play an important role in their ability to stimulate sperm movement.

In conclusion, the present report demonstrates that rat caput spermatozoa do not exhibit mature-cell motility when exposed to cauda epididymal fluid and cyclic adenine nucleotides. Additionally, the motility stimulation observed in caput cells diluted with T appeared to be an effect specific to xanthine PDIs which may not be due simply to their ability to inhibit phosphodiesterase activity. While epididymal proteins, protein-sperm cell membrane interactions, and subsequent intracellular molecular events are probably very important

to epididymal maturation of spermatozoa and to the acquisition of mature sperm cell motility, the present evidence contraindicates the presence of a specific rat epididymal FMP similar to that previously found by others in the bull. The acquisition of mature-cell motility is probably the result of many separate but interlocking events during the epididymal maturation of rat spermatozoa.

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References

- Brandt H, Acott TS, Johnson DJ, Hoskins DD. Evidence for an epididymal origin of bovine sperm forward motility protein. *Biol Reprod* 1978; 19:830-835.
- Brandt H, Hoskins DD. A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. *J Biol Chem* 1980; 255:982-987.
- Casillas ER, Elder CM, Hoskins DD. Adenylate cyclase activity of bovine spermatozoa during maturation in the epididymis and the activation of sperm particulate adenylate cyclase by GTP and polyamines. *J Reprod Fertil* 1980; 59:293-302.
- Frenkel G, Peterson RN, Freund M. The role of adenine nucleotides and the effect of caffeine and dibutyryl cyclic AMP on the metabolism of guinea pig epididymal spermatozoa. *Proc Soc Exptl Biol Med* 1973; 144:420-425.
- Garbers DL, First NL, Gorman SK, Lardy HA. The effects of cyclic nucleotide phosphodiesterase inhibitors on ejaculated porcine spermatozoan metabolism. *Biol Reprod* 1973a; 8:599-606.
- Garbers D, First NL, Lardy HA. The stimulation of bovine epididymal sperm metabolism by cyclic nucleotide phosphodiesterase inhibitors. *Biol Reprod* 1973b; 8:589-598.
- Hoskins DD, Brandt H, Acott TS. Initiation of sperm motility in the mammalian epididymis. *Fed Proc* 1978; 37:2534-2542.
- Hoskins DD, Hall ML, Munsterman D. Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. *Biol Reprod* 1975; 13:168-178.
- Levin RM, Greenberg SH, Wein AJ. Quantitation of effects of caffeine on sperm motility and cyclic AMP phosphodiesterase. Abstracts of the Annual Meeting of the American Urological Association 1981; 142.
- Morton BE, Fraser CF, Sagadraca R. Initiation of hamster sperm motility from quiescence: effect of conditions upon flagellation and respiration. *Fertil Steril* 1979; 32:222-227.
- Morton BE, Sagadraca R, Fraser CF. Sperm motility within the mammalian epididymis: species variations and correlation with free calcium levels in epididymal plasma. *Fertil Steril* 1978; 29:695-698.
- Nelson L, Young MJ, Gardner ME. Sperm motility and calcium transport: a neurochemically controlled process. *Life Sci* 1980; 26:1739-1749.
- Turner TT. On the epididymis and its function. *Invest Urol* 1979; 16:311-321.
- Turner TT, Howards SS. Factors involved in the initiation of sperm motility. *Biol Reprod* 1978; 18:571-578.
- Turner TT, D'Addario D, Howards SS. Further observations on the initiation of sperm motility. *Biol Reprod* 1978; 19:1095-1101.
- Wyker R, Howards SS. Micropuncture studies of the motility of rete testis and epididymal spermatozoa. *Fertil Steril* 1977; 28:108-112.