BIOLOGY OF REPRODUCTION 32, 489–500 (1985)

Evidence for a Role for Cellular Alkalinization in the Cyclic Adenosine 3′,5′-Monophosphate-Mediated Initiation of Motility in Bovine Caput Spermatozoa

S. VIJAYARAGHAVAN,[1] L. M. CRITCHLOW and D. D. HOSKINS

Department of Reproductive Biology and Behavior
Oregon Regional Primate Research Center
Beaverton, Oregon 97006

ABSTRACT

Bicarbonate ion, the local anesthetics procaine and dibucaine, and the ionophores monensin and nigericin have been shown to markedly increase the ability of agents that elevate cyclic adenosine monophosphate (cAMP) levels to initiate motility in bovine caput spermatozoa. A number of other weak bases, including theophylline, D-600 and dipyridamole, elevate cAMP levels maximally in caput sperm at low levels but induce motility only at high levels. These compounds thus appear to have a dual role in the initiation of motility, i.e., they elevate both cAMP levels and internal pH. Confirmation of this view was provided by the demonstration that bicarbonate ion and procaine permit initiation of motility by theophylline, D-600 and dipyridamole at markedly reduced levels. Also, forskolin (a neutral adenylate cyclase activator) elevates cyclic AMP levels in caput sperm but initiates motility only in the presence of bicarbonate or procaine, and the membrane-permeant cAMP analogue 8-bromo-cAMP is capable of inducing motility only in the presence of bicarbonate. Thus, motility in caput sperm is induced only under conditions that elevate both intracellular cAMP and pH, whereas caudal sperm motility is stimulated by an elevation of either cAMP or pH. These data suggest that the epididymal development of motility requires a maturational increase in internal pH. This suggestion was confirmed by direct measurement of the internal pH of caput and caudal sperm; the internal pH of the former was found to be 5.84 ± 0.1 and the latter 6.27 ± 0.05.

INTRODUCTION

Mammalian sperm acquire the capacity for motility during transit of the epididymis (Bedford, 1975). Washed sperm isolated from the caput epididymidis are essentially immotile, although some may display a twitching motion and those isolated from the caudal region show vigorous progressive motion (Hoskins et al., 1978, 1979). The biochemical basis for the acquisition of the capacity for motility is poorly understood. However, it is known that cyclic adenosine monophosphate (cAMP) levels in sperm from a number of species (Hoskins et al., 1974; Amann et al., 1982; Goh, 1983) increase during epididymal transit; that a number of cAMP phosphodiesterase (PDE) inhibitors that elevate intrasperm levels of cAMP induce an uncoordinated pattern of motility in bovine caput sperm; and that this pattern is converted to the directional pattern of mature caudal sperm by addition of crude preparation of a "forward motility protein" (FMP) present in epididymal fluids (Hoskins et al., 1975; Brandt et al., 1978). Cyclic AMP PDE inhibitors also stimulate motility in mature submotile caudal sperm. It thus appears that elevation of cAMP levels in sperm is responsible for both the development of motility in immature sperm and the maintenance of motility in mature sperm.

Acceptance of the view that cAMP is involved in the development of motility has been marred, however, by the observation that inordinately high levels of PDE inhibitors are required to stimulate motility in caput sperm in vitro. These levels are 30- to 40-fold higher than those required to stimulate motility in mature, submotile caudal sperm. Moreover, this activation of caput sperm is seen only after a pronounced lag phase, and the membrane-permeable cAMP analogue dibutyrl cAMP does not initiate motility (Hoskins et al., 1975). Thus, it has not yet been possible to initiate motility in vitro in bovine caput sperm under

Accepted October 11, 1984.
Received June 12, 1984.
[1] Reprint requests.

conditions that might be construed as physiologic.

In this paper, we provide evidence that one reason for this failure to initiate motility in vitro has been that yet another factor, an increase in intracellular pH, is required before an increase in the intrasperm level of cAMP—however brought about—can be manifested as motility initiation. We show that all of the previously reported anomalies associated with in vitro motility initiation in bovine caput sperm vanish if the in vitro activation medium contains bicarbonate or agents that lead to an elevation of intracellular pH. It appears that the efficacy of high levels of certain phosphodiesterase inhibitors in initiating motility is due both to their ability to elevate cAMP levels and to their basicity (or ability to elevate intracellular pH by other means). Caudal sperm motility, on the other hand, can be stimulated by either an elevation in intracellular pH or an increase in cellular cAMP levels, an observation suggesting that caudal sperm have a more alkaline cytoplasmic environment than caput sperm. This has been confirmed by measurement of the internal pH of caput and caudal sperm.

Alkalinization of cells is known to activate a wide variety of physiologic processes, including fertilization (Roos and Boron, 1981). The impetus for our present study was the recent demonstration by Garbers et al. (1983) that bicarbonate ion, in the presence of calcium, markedly elevates cAMP levels in mature guinea pig sperm. Other recent studies have shown that an increase in intracellular pH, induced by membrane-permeable bases, stimulates motility and respiration in bovine caudal sperm (Babcock et al., 1983) and that an increase in intracellular pH is involved in the activation of sea urchin (Lee et al., 1983) and rat caudal sperm (Wong et al., 1981).

The mechanism by which pH changes stimulate motility is not known. It is also not known whether intrasperm changes in pH and levels of cAMP are independent aspects of motility control or whether they are related. The results of our study have shed light on this question by showing that bicarbonate and membrane-permeable weak bases do not act primarily to modulate cAMP levels in caput sperm. Instead, we found evidence that bicarbonate ion and other weak bases markedly alter the ability of caput sperm to respond to agents that elevate cAMP levels, such as theophylline, D-600, dipyridamole, forskolin, and the cAMP analog 8-bromo-cAMP (8-Br-cAMP). However, stimulation of motility in mature caudal sperm does not require the addition of weak bases and can be accomplished by the addition of low levels of phosphodiesterase inhibitors, forskolin, or 8-Br-cAMP. We suggest, therefore, that alkalinization of flagellar cytosol is a necessary step in the development of the capacity for motility in bovine sperm during epididymal transit.

MATERIALS AND METHODS

Bovine testicles were obtained from a local slaughterhouse and delivered to the laboratory within 2 h of slaughter. Caput spermatozoa were recovered, as previously described (Hoskins et al., 1975), from the distal caput area of the epididymis [region 2 in the schema of Casillas (1973)]. Caudal spermatozoa were obtained by extrusion from the distal caudal epididymidis as described by Henle and Zittle (1942). For the studies on internal pH, caput and caudal sperm were prepared from the same testes. Usually sperm from 5–10 testes were pooled. The isolation and washing buffer for both sperm types consisted of 42 mM KCl, 103 mM NaCl, 5 mM $MgSO_4$, 10 mM KH_2PO_4, and 10 mM tris(hydroxymethyl)aminomethane, pH 7.5 (CESD). After being layered onto 10 ml of 8% Ficoll, sperm were washed in CESD by centrifugation first for 5 min at 500 × *g* and then for 5 min at 1150 × *g* (Harrison, 1976). Sperm pellets were resuspended in CESD containing 10 mM glucose and 5 mg of bovine serum albumin/ml and used within 15 min after resuspension. Fifteen μl/ml of bovine seminal plasma (supernatant of semen centrifuged at 800 × *g* for 30 min), as a source of FMP, were included in the incubation medium in motility experiments with caput sperm (Brandt et al., 1978).

Stock bicarbonate-CESD solutions were prepared by substitution of 100 mM $NaHCO_3$ for NaCl in the CESD. The desired level of bicarbonate in experimental tubes was obtained by appropriate dilution in CESD of bicarbonate-CESD, prepared fresh before each experiment. Individual experiments involving bicarbonate ion of less than 5 min duration were conducted in open tubes; experiments requiring longer time periods involved incubation under an atmosphere of 5% CO_2 and 95% air. Experiments that required measurement of cAMP levels were carried out at 30°C, and those involving motility determinations were conducted at 37°C. Cyclic nucleotide levels are not significantly different when measured at 30° or 37°C (Vijayaraghavan, unpublished; Hammerstedt and Hay, 1980).

Motility Measurement

Motility was determined by two methods. The first was a visual evaluation by three observers with a phase-contrast microscope. Motility was rated on a fractional scale of 0–10; the numerator denoted the percentage of sperm showing any type of motion, and the denominator denoted the percentage of sperm showing forward progression. Percent motility and average velocity were measured by the videomicro-

graphic procedure of Katz and Overstreet (1981). Measurements were on at least 50 sperm randomly selected from recordings of at least 5 fields.

Cyclic AMP Determination

Cyclic AMP was measured as described by Steiner et al. (1972) after 2'-O-acetylation of the nucleotide (Harper and Brooker, 1975). Antiserum was raised in rabbits against a 2'-*O*-succinyl bovine serum albumin conjugate and was used at a final dilution of 1:60,000. The radiolabeled ligand was 2'-*O*-monosuccinyl-cAMP-tyrosine methyl ester (Sigma Chemical Co., St. Louis, MD) iodinated with ^{125}I with chloramine-T (Greenwood et al., 1963). Details of the specificity of the antiserum and sensitivity of the assay have been reported (Ellinwood et al., 1980).

Measurement of Cellular pH

Sperm cytosolic pH was measured with the fluorescent probe carboxyfluorescein, generated intracellularly during a preincubation of the cells with carboxyfluorescein diacetate. The technique was essentially the same as that reported by Babcock (1983), except that fluorescence instead of absorbance was measured. Carboxyfluorescein diacetate was synthesized as reported by Thomas et al. (1979), and the product was crystallized from acetone. Caudal or caput sperm were removed as described, and washed twice in buffer containing 120 mM NaCl, 1 mM $MgCl_2$ and 30 mM *N*-morpholino propane sulfonic acid, pH 7.4. About 2.5×10^7 cells/ml were suspended in loading buffer containing 120 mM NaCl, 1 mM $MgCl_2$, 30 mM *N*-morpholine ethane sulfonic acid (MES), pH 6.1, and 5 μM carboxyfluorescein diacetate (added from 1 mM stock solution prepared in dimethyl sulfoxide) and incubated for 20 min at 25°C. At the end of the incubation period, 2-ml aliquots were removed and the cells were formed into pellets by centrifugation at 2000 × *g* for 5 min. The pellets were suspended in buffer (120 mM NaCl, 1 mM $MgCl_2$ and 50 mM MES) with pH values ranging from 5.8 to 7.2. Fluorescence was measured at excitation wavelengths of 495 nm and 450 nm, and emission was measured at 515 nm before and after addition of 100 μg of digitonin (prepared as a stock solution in dimethyl sulfoxide). Differences in the fluorescence intensity ratios before and after digitonin treatment were plotted as a function of external pH. Interpolation to a zero fluorescence ratio difference gave the value of the internal pH of the cells.

Theophylline, dipyridamole, procaine, dibucaine, 8-Br-cAMP and digitonin were purchased from the Sigma Chemical Company, St. Louis, MO. D-600 and forskolin were obtained from Calbiochem-Boehring, San Diego, CA and the carboxyfluorescein from Kodak, Rochester, NY. All other chemicals were of the highest purity available. Dipyridamole, dibucaine, forskolin and D-600 were dissolved in 100% ethanol and added to individual incubation tubes so that the final ethanol concentration did not exceed 0.1%. Corresponding amounts of ethanol were used in control incubation tubes.

RESULTS

Effects of Bicarbonate on Bovine Spermatozoal Motility and the Sensitivity of Caput Sperm to Motility Induction by Theophylline

Prompted by the observation that bicarbonate and calcium have dramatic stimulatory effects on cAMP in guinea pig spermatozoa (Garbers et al., 1983), we first studied the effects of these compounds on motility and cAMP levels in bovine caput spermatozoa. Bicarbonate ion was found to have a moderate but inconsistent stimulatory effect on motility of spermatozoa. Figure 1 shows an experiment in which approximately 30% of the cells were stimulated at a bicarbonate concentration of 15 mM or higher. In a number of experiments, the stimulatory effect varied considerably among sperm preparations and the percentage of induced motility ranged from 0% to 30%.

However, addition of theophylline (1 mM) in the presence of bicarbonate consistently induced a high level of motility, which was reflected in both the percentage of motile cells and their average velocity (Table 1). Theophylline by itself at this concentration only marginally increased the percentage of motile caput sperm. In contrast, caudal sperm motility was stimulated markedly by either bicarbonate or theophylline (Table 1). The addition of external calcium (0.5 mM) had no effect on the bicarbonate stimulation of motility in either cell type (data not shown).

The effect of increasing levels of theophylline in the presence of bicarbonate on the motility of caput sperm was examined in more detail, and the results are presented in Table 2. The data show that, in the absence of bicarbonate (20 mM), 20 mM theophylline is required to induce maximum motility in caput spermatozoa, whereas only 1–2 mM is required when bicarbonate is present. The data also show that the level of motility achieved even in the presence of 20 mM theophylline is further enhanced by bicarbonate. The effects of 8-Br-cAMP (1 mM) on motility initiation in the absence and presence of bicarbonate were also tested. The results of a representative experiment are also shown in Table 2 (Exp. II). This cAMP analogue had no effect by itself, but dramatically stimulated motility in the presence of bicarbonate. The marginal effect of bicarbonate by itself on motility in both experiments was similar to that shown in Figure 1.

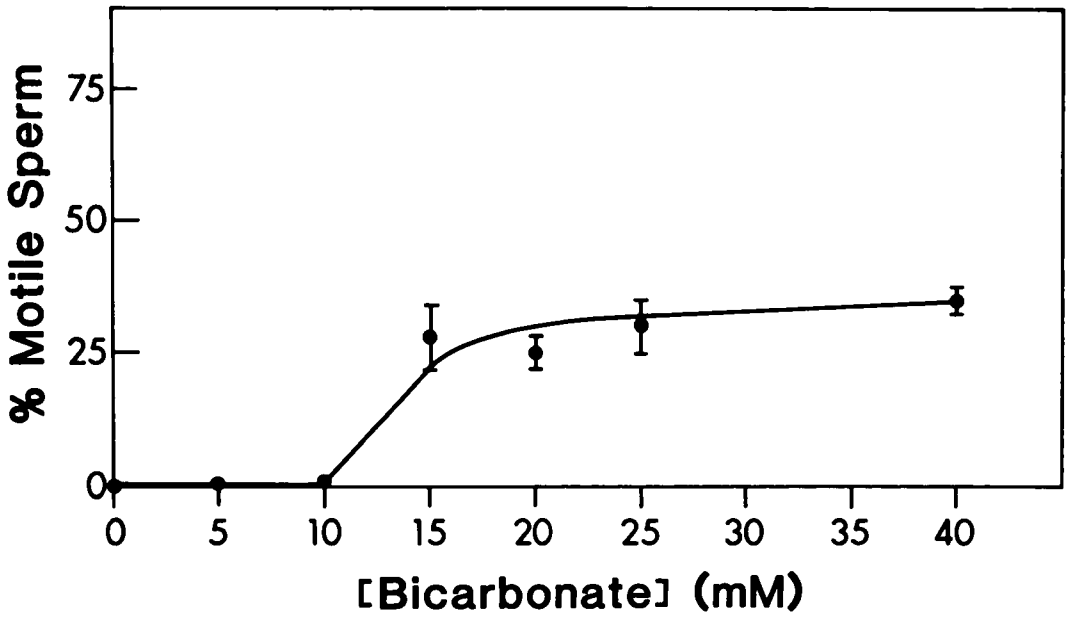

FIG. 1. Effect of bicarbonate concentrations on motility induction in bovine caput sperm. Caput sperm were incubated at 37°C with the indicated amounts of $NaHCO_3$ replacing NaCl in CESD. Motility was quantitated by videomicrography at 5 min. Mean % motility ± SEM was derived from values obtained from 5 different fields.

Effects of Bicarbonate and Theophylline on Cyclic AMP Levels in Bovine Caput Spermatozoa

The effects of bicarbonate (20 mM) and varying concentrations of theophylline on cAMP levels and motility in caput spermatozoa are shown in Figure 2. Bicarbonate ion alone increased the cAMP level marginally (in fact, from 120 to 150 pmol/10^9 sperm) and motility to a similar, modest degree (from 1/0 to 3/1). In the presence of increasing levels of theophylline, however, cAMP levels were correspondingly increased (Fig. 2). In the absence of bicarbonate, motility induction reached a maximum at a theophylline concentration of 10–20 mM, and in its presence maximum motility was

TABLE 1. Effects of bicarbonate on motility in bovine spermatozoa.[a]

Epididymal origin of sperm	Additions	Motility parameters	
		% Motile[b]	Average velocity[c]
Caput	None (control)	0	0
	Bicarbonate (25 mM)	36.0 ± 7.0	51.6 ± 4.7
	Theophylline (1 mM)	15.0 ± 3.5	–
	Bicarbonate (25 mM) + theophylline (1 mM)	67.0 ± 5.0	66.7 ± 7.5
Cauda	None (control)	28.0 ± 7.3	135.0 ± 9.7
	Bicarbonate (25 mM)	88.0 ± 3.7	154.3 ± 7.0
	Theophylline (1 mM)	88.0 ± 3.0	162.1 ± 6.0

[a]Bovine caput or caudal sperm were incubated at 37°C with the indicated additions. The 8-μl aliquots of the incubation mixture were removed between Minutes 5 and 15 for quantification of motility by videomicrography.

[b]Average plus SEM of values derived from 5 fields; at least 10 sperm were counted per field.

[c]The micrometer per second values are averages plus SEM of velocities of motile sperm from 5 fields; at least 10 motile sperm were selected per field.

TABLE 2. Effects of theophylline concentration and 8-bromo-cAMP on motility induction in bovine caput sperm.[a]

Experiment Number	Treatment	Motility −Bicarbonate	Motility +Bicarbonate
I	None (control)	0/0	1/0
	Theophylline 1 mM	2/1	7/6
	2 mM	2/2	8/6
	4 mM	3/3	8/6
	6 mM	5/3	8/6
	10 mM	6/4	8/6
	20 mM	6/5	8/6
II	None (control)	0/0	3/1
	8-Br-cAMP 1 mM	0/0	8/4

[a]Bovine caput sperm were incubated with the indicated additions with or without bicarbonate (20 mM). Motility was measured by visual estimation between Minutes 5 and 25, as described under "Materials and Methods."

achieved at much lower (1–2 mM) levels of theophylline (see also Table 2). Low levels (0–5 mM) of theophylline increased cAMP levels to nearly the same degree in the presence and absence of bicarbonate, but motility at these theophylline concentrations was markedly less in the absence of bicarbonate (lower curve, Fig. 2).

Effects of Forskolin, D-600, and Dipyridamole on Cyclic AMP Levels and Motility in Bovine Spermatozoa

To see if the permissive effect of bicarbonate on motility induction by theophylline was specific to the phosphodiesterase inhibitor, we tested compounds with varied chemical structures and properties for their ability to induce

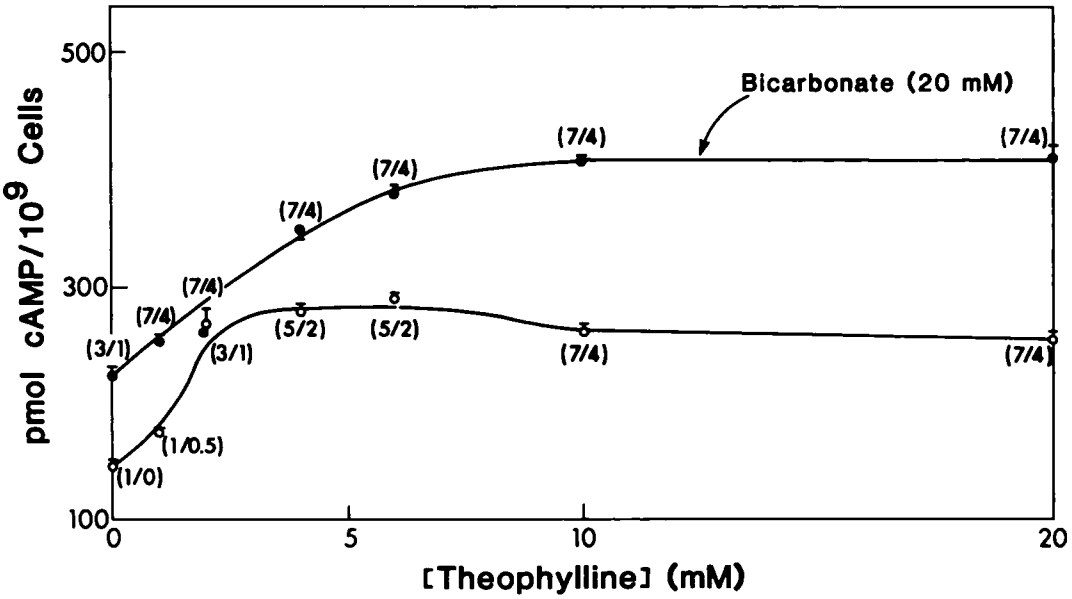

FIG. 2. Motility induction and cAMP levels (mean ± SEM) by theophylline concentration with or without bicarbonate in bovine caput sperm. Sperm were incubated with the indicated concentrations of theophylline with or without bicarbonate, and at 5 min 200-μl triplicate aliquots were removed for cAMP assay. Motility (in parentheses) was measured by visual estimation.

motility. Forskolin, a diterpene, is known to elevate cAMP in other tissues (Seamon and Daly, 1981) through activation of adenylate cyclase. Dipyridamole, a (2,2′,2″,2‴-(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyldinitrilo)tetraethanol, and D-600, a (α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methyl-amino]propyl]-3,4,5-trimethoxy-α-(1-methylethyl)benzenacetonitrile), are both weak bases; the former is known to inhibit adenosine transport (Huang and Daly, 1974) and cAMP phosphodiesterase (Amer and Kreighbaum, 1975), and the latter is a calcium channel blocker (Triggle, 1982). Time courses for changes in cAMP levels and motility ratings in caput sperm in the presence of these three agents are shown in Figure 3. All three compounds increased cAMP levels significantly at 5 and 10 min, at the concentrations indicated, in both the absence and the presence of bicarbonate. Significantly, motility induction (fractional numbers in parentheses) by these compounds was low in the absence of bicarbonate and high in its presence.

D-600 at the concentration used in the experiment shown in Figure 3 by itself did not induce motility. Since the compound is a weak base and was able to elevate cyclic AMP, we reasoned that it should induce motility in caput sperm at higher concentrations, in a manner similar to theophylline. Therefore, its effect over a wide concentration range (0—800 μM) was examined in greater detail (Fig. 4). The data (lower curve) show that D-600 alone caused a progressive increase in cAMP and motility, and that nucleotide elevation reached a maximum at 400 μM D-600. As was the case with theophylline (Fig. 2), maximum motility induction occurred at levels (> 600 μM) greater than that required to achieve the maximum cAMP elevation. The discordance between the extent of cAMP elevation and motility induction is exemplified again in Figure 4 (upper curve), which shows the cAMP levels obtained with various levels of D-600 in the presence of 1 mM theophylline. Although cAMP remained maximally elevated at all concentrations of D-600, maximum motility induction occurred only at concentrations greater than 200 μM. Dipyridamole, like D-600, also induced maximum motility in caput sperm

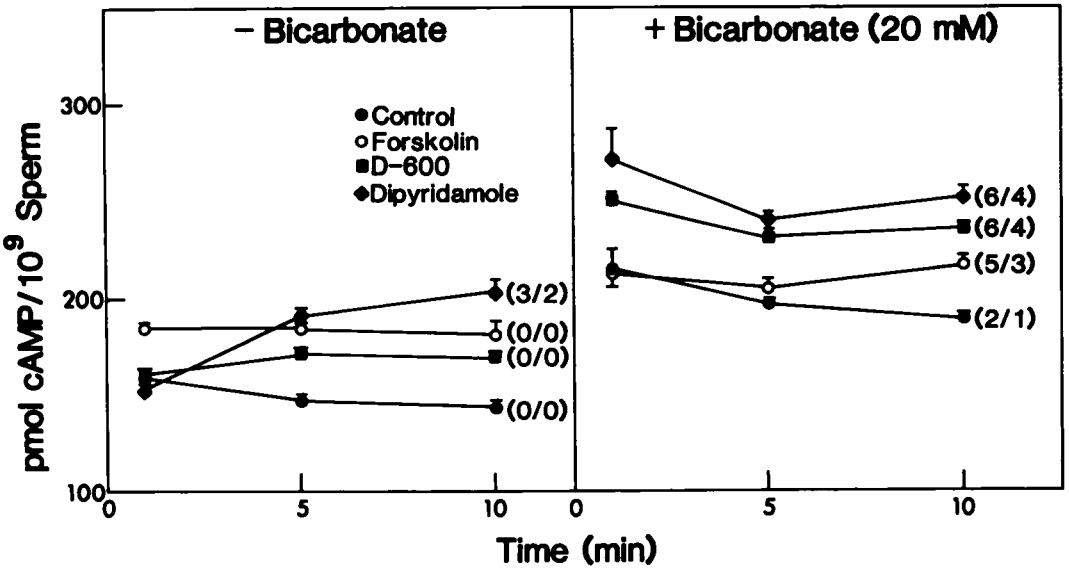

FIG. 3. Stimulation of cAMP levels (mean ± SEM) in caput sperm with or without bicarbonate (20 mM). Sperm were incubated with the indicated additions and at the times indicated 200-μl triplicate aliquots were removed for cAMP assay. Motility (in parentheses) was measured at 5—15 min using an identical set of incubation tubes. All points at 5 and 10 min (except forskolin and bicarbonate at 5 min) were statistically significant ($P<0.001$) compared to respective controls.

at a higher concentration than that used in the experiment shown in Figure 3 (data not included).

Effects of Weak Bases and Ionophores on Motility in Bovine Spermatozoa

The data shown in Figures 2, 3, and 4 indicated that, in addition to an elevation in the level of cAMP, alkalinization of internal pH might be required for motility induction in caput sperm. Consequently, different agents expected to raise the cytosolic pH in cells were used to study motility induction. Figure 5 shows the effects of forskolin, D-600, and theophylline on motility initiation and cAMP levels in caput sperm in the presence and absence of the local anesthetic procaine. Overall, procaine had effects similar to those of bicarbonate (Fig. 3) in that it permitted low levels of these agents, which by themselves elevate cAMP, to induce motility. Procaine by itself elevates cAMP at 5 min and induces some motility (2/1).

Table 3 shows the effects of procaine, dibucaine, ammonium chloride, monensin and nigericin with and without 1 mM theophylline on motility induction in caput sperm and the effect of these agents on motility in caudal sperm. The concentrations of procaine (17 mM), dibucaine (1 mM), monensin (0.4 μM) and nigericin (1.7 μM) were optimum concentrations required for maximum induction of motility in caput sperm in the presence of 1 mM theophylline (data not shown). Also, monensin, nigercin and ammonium chloride (40 mM) at concentrations comparable to those used here (Table 3) have been shown to alkalinize the cytosolic environment of bovine caudal

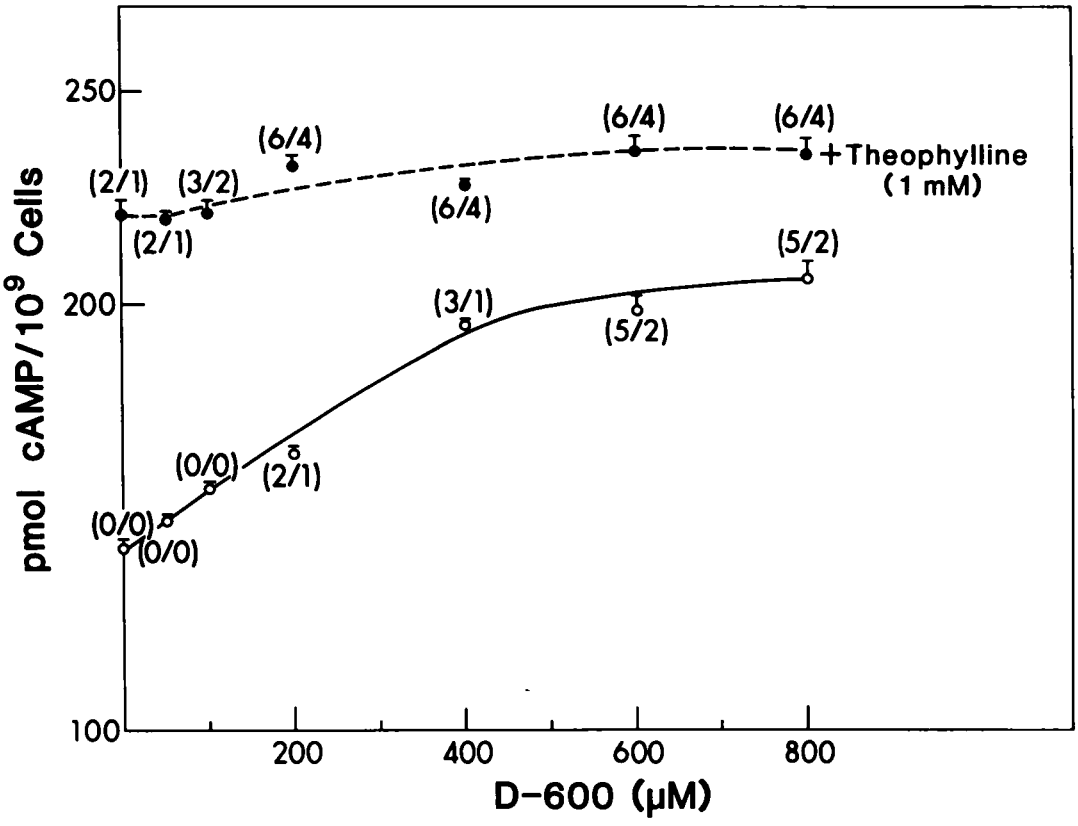

FIG. 4. Cyclic AMP (cAMP) levels (mean ± SEM) in bovine caput sperm treated with D-600 concentrations with or without theophylline. Sperm were incubated with the indicated additions and at 5 min 200-μl triplicate aliquots were removed for cAMP assay. Motility (in parentheses) was measured between Minutes 5 and 15 using an identical set of incubation tubes.

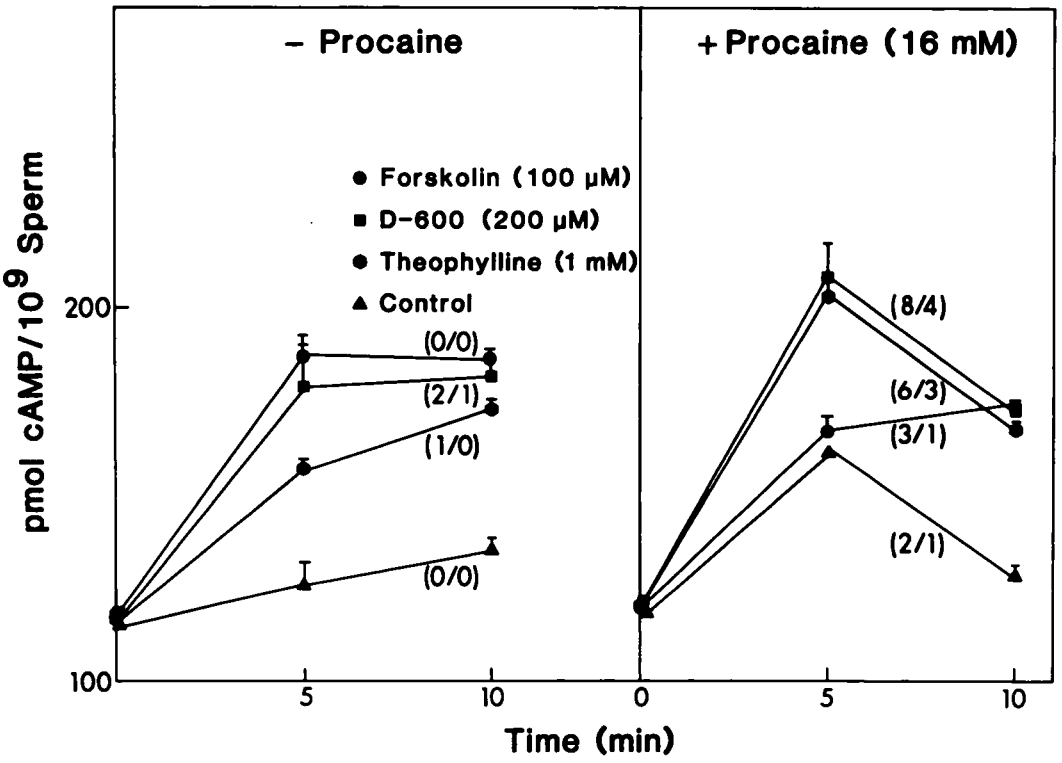

FIG. 5. Cyclic AMP (cAMP) levels (average ± SEM) in caput sperm with or without procaine. Incubation condition similar to that described under Figure 3. Motility (in parentheses) was determined between 5 and 10 min. All points at 5 and 10 min (except forskolin and procaine at 5 min) were statistically significant ($P<0.001$) compared to respective controls.

sperm (Babcock, 1983; Babcock et al., 1983). Procaine, dibucaine and ammonium chloride acted in a manner similar to that of bicarbonate, i.e., in their presence low levels (1 mM) of theophylline induced motility in caput sperm. Although dibucaine by itself induced some motility, which was enhanced in the presence of theophylline, none of the remaining agents showed an effect on motility by themselves. Caudal sperm motility, on the other hand, was stimulated by all three bases. The sodium ionophore monensin also behaved like the weak bases, i.e., permitted motility initiation (24%) in caput sperm by theophylline. This was a weak effect compared to that obtained with procaine or dibucaine, but it was still significant considering the fact that theophylline by itself was inactive. Monensin also stimulated motility by itself in caudal sperm. Caput sperm suspended in a K^+ buffer were stimulated by 1 mM theophylline, and nigericin marginally increased the effect. Caudal sperm suspended in K^+ buffer displayed considerably more motility than cells in a Na^+ buffer or CESD. Nigericin appeared to have no effect on motility in caudal sperm. In sum, it appeared that agents that elevated the intracellular pH by a simple permeation (e.g., procaine, dibucaine and ammonium chloride) and those that elevated the pH by a sodium or potassium exchange with protons (e.g., monensin and nigericin) caused expression of motility in theophylline-treated sperm.

Measurement of Internal pH of Bovine Epididymal Spermatozoa

Since caput sperm required an elevation of internal pH in addition to cAMP response, it appeared very likely that the cytosolic pH of caput sperm was lower than that of caudal sperm. We therefore measured the internal pH of caput and caudal sperm. Figure 6 shows the results of a typical experiment. The method yielded an internal pH value for caput sperm 0.4 units less than the value for caudal sperm.

TABLE 3. Effect of weak bases and ionophores on motility in bovine spermatozoa.[a]

Treatment	Theophylline (1 mM)	% Motility[b] Caput	Caudal
1. None (control)	–	0	44.0 ± 5.0
	+	0	–
Procaine (17 mM)	–	0	95.0 ± 3.3
	+	42.0 ± 6.3	–
2. None (control)	–	0	8.0 ± 3.3
	+	0	–
Dibucaine (1 mM)	–	30.0 ± 6.3	62.0 ± 6.3
	+	54.0 ± 3.2	–
3. None (control)	–	0	36.7 ± 9.5
	+	0	–
NH_4Cl (40 mM)	–	0	70.0 ± 6.1
	+	23.3 ± 9.5	–
4. None (control)	–	0	53.0 ± 6.7
	+	0	–
Monensin (0.4 μM)	–	0	95.0 ± 5.0
	+	23.6 ± 5.9	–
5. None (control)	–	0	92.0 ± 3.3[c]
	+	32.0 ± 5.3	–
Nigericin (1.7 μM)	–	0	52.5 ± 11.3
	+	46.0 ± 6.7	–

[a]Experiments 4 and 5 involved a Na^+ and K^+ buffer, respectively, (i.e., NaCl or KCl replacing KCl or NaCl in CESD). The rest of the experiments involved standard incubation conditions described in "Materials and Methods." Caudal or caput sperm were incubated at 37°C with the indicated additions and 8 μl aliquots were removed at 0–5 min for measurement of motility by videomicrography.

[b]Average plus SEM of values derived from 5 fields.

[c]Percent motility of caudal sperm suspended in the standard incubation buffer (CESD) was 50.0 ± 3.5 in this experiment.

The values of the internal pH averaged over eight independent determinations were 5.84 ± 0.1 and 6.27 ± 0.05 for caput and caudal sperm, respectively.

DISCUSSION

The initial impetus for our study was the observation that bicarbonate and calcium ions act synergistically to release cAMP levels in mature guinea pig sperm (Garbers et al., 1983). Bicarbonate and calcium had little or no effect on cAMP levels in bovine epididymal sperm, but our observation that bicarbonate permitted motility stimulation in caput sperm by low concentrations of theophylline (Table 1 and Fig. 2) led to formulation of two questions. First, why are such high concentrations of phosphodiesterase inhibitors required to induce motility in caput sperm? Second, how does bicarbonate increase the sensitivity of caput sperm to motility stimulation by theophylline? The data in Figure 1 provide a clue to the answers.

These data show that, in spite of maximal elevation of cAMP by lower concentrations of theophylline, maximal motility induction occurs only at higher concentrations and that bicarbonate permits motility induction at considerably lower theophylline concentrations. Poor permeability or an insufficient cAMP response, as previously assumed, cannot be the explanation for the failure of low levels of theophylline to induce motility in caput sperm. Thus, it is likely that both theophylline at a high concentration (20 mM) and bicarbonate alter the same properties in the cell (in addition to cAMP) required for motility to be expressed.

We suggest that this property is intracellular pH. Bicarbonate is known to increase intracellular pH in other cells, including squid axon and barnacle muscle (Boron and DeWeer, 1976; Boron et al., 1979), and theophylline, a weakly basic compound (pK_a = 8.7), would be expect-

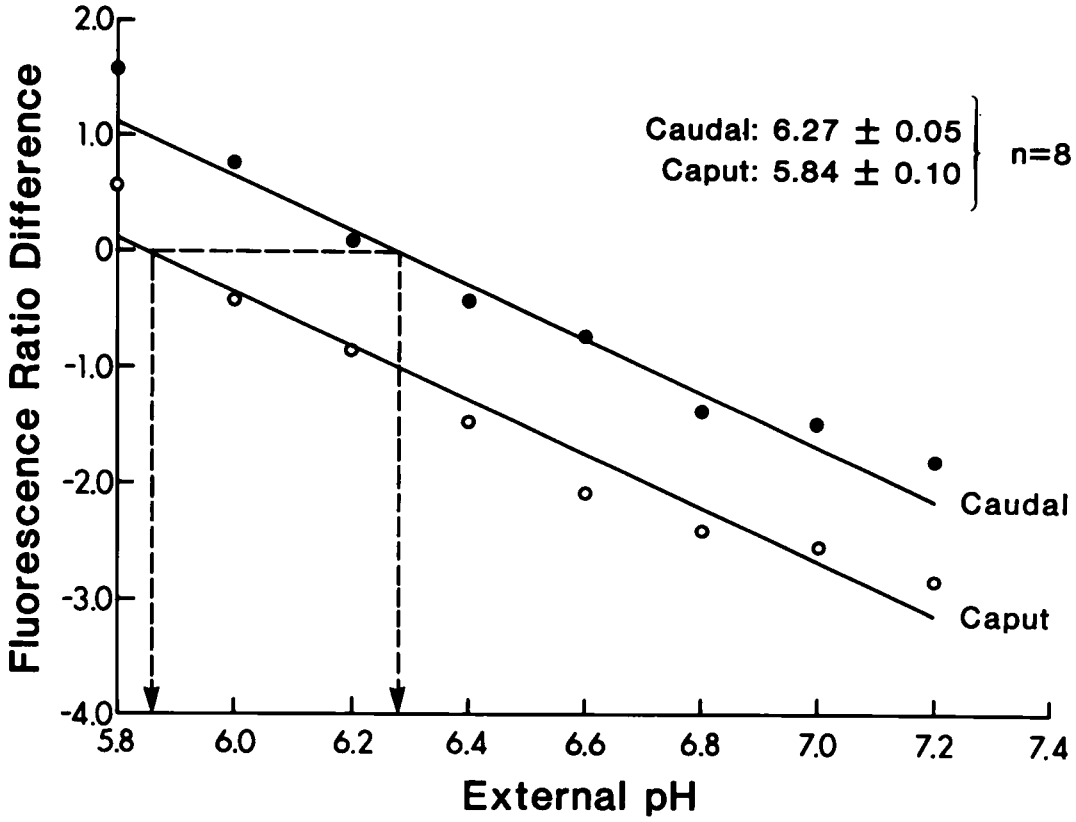

FIG. 6. Null point determination of the cytosolic pH of bovine sperm. Twice-washed caput or caudal sperm (2.5×10^7 cells/ml) were incubated in loading buffer containing 5 μM carboxyfluorescein diacetate for 20 min at 25°C. After pelleting the cells (2-ml aliquots) by centrifugation (2000 × *g*, 5 min) the cells were suspended in 2 ml of 120 mM NaCl, 1 mM $MgCl_2$, 5 mM glucose, and 50 mM sodium morpholinoethane sulfate at the indicated pH. Fluorescence intensity was measured at 495 nm and 450 nm excitations and at 550 nm emission wavelengths, before and after addition of 100 μg/ml of digitonin. The differences of fluorescence intensity ratios before and after digitonin addition were plotted as a function of external pH. The lines were fit by a least-squares linear regression analysis.

ed to increase the intracellular pH, as do other bases (Roos and Boron, 1981). It seems likely that these compounds increase the intracellular pH in caput sperm under the conditions used in our motility experiments. The data (Figs. 3 and 4) support this possibility: D-600 and dipyridamole, both bases, induce motility at high concentrations; forskolin and 8-Br-cAMP (Table 1) do not by themselves induce motility, but do in the presence of bicarbonate. The local anesthetics procaine and dibucaine and ammonium chloride mimic the effects of bicarbonate on motility induction (Fig. 5 and Table 3). Ammonium chloride has been used as an agent to induce alkalinization in a wide range of cells (Roos and Boron, 1981) and recently has been shown to cause alkalinization in bovine caudal sperm (Babcock, 1983). The weak bases procaine (pK_a = 8.95) and dibucaine have also been shown to raise intracellular pH (Roos and Boron, 1981). Thus, the effect of the weak bases and bicarbonate on caput sperm indicates that motility induction requires alkalinization of internal pH, in addition to cAMP elevation. However, it is possible that procaine and dibucaine could have other effects in sperm in addition to elevation of internal pH, since these agents by themselves induce some motility and elevate cyclic AMP in caput sperm (Table 3, Fig. 5).

The sodium ionophore monensin, when added to cells suspended in an appropriate buffer so that proton and sodium gradients exist in opposite directions, promotes a sodium-

proton exchange that dissipates both the gradients. In fact, it has been demonstrated that monensin added to cells suspended in high-sodium buffers more alkaline than the intracellular environment induces alkalinization of the intracellular pH of bovine caudal sperm (Babcock, 1983). Nigericin acts in a similar fashion when sperm are suspended in buffers containing a high concentration of K^+ (Babcock, 1983). Our data (Table 3) on the effects of monensin and nigericin on motility initiation in the presence of theophylline are consistent with the idea that alkalinization of caput sperm is required before an increase in the intracellular level of cAMP can be made manifest. The data in Table 3 also indicate that high-K^+ buffers (pH 7.5) act to alkalinize the cellular pH. Indeed, Babcock et al. (1983) have recently provided evidence for a potassium-mediated alkalinization of the pH of bovine caudal sperm.

The stimulation of motility in caput sperm, under conditions that increase both intracellular cAMP and pH, is suggestive of a developmental increase in cellular pH. The fact that caudal sperm motility is stimulated by a simple elevation of cAMP supports this conclusion. A developmental increase in cAMP levels during epididymal transit is already well established (Hoskins et al., 1975; Amann et al., 1982). Our study has provided evidence that a developmental increase in cellular pH, in addition to a cAMP elevation, is required for the expression of motility. On the basis of our preliminary data on the internal pH, caudal sperm have a more alkaline intracellular environment than caput sperm. Using essentially the same method of measurement used in this study, Babcock (1983) reported a value of 6.5 for the internal pH of bovine caudal sperm. This value is 0.2 units higher than that reported here. The reason for this discrepancy is not known. This observation needs to be placed on a firmer footing by means of other, independent methods of intracellular pH measurement. Nevertheless, we have presented strong evidence that intracellular alkalinization indeed occurs during epididymal development in bovine sperm and that optimum expression of motility in caput sperm in vitro requires an elevation of both internal pH and cyclic nucleotide levels.

ACKNOWLEDGMENTS

The work described in this article, Publication No. 1379 of the Oregon Regional Primate Research Center, was supported by NIH grants HD-11982 and RR-00163. We thank Dr. David L. Garbers for providing the manuscript of his data prior to publication (Garbers et al., 1983).

REFERENCES

Amann, R. P., Hay, S. R. and Hammerstedt, R. H. (1982). Yield, characteristics, motility and cAMP content of sperm isolated from seven regions of ram epididymis. Biol. Reprod. 27:723–733.

Amer, M. S. and Kreighbaum, W. E. (1975). Cyclic nucleotide phosphodiesterases: properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. J. Pharm. Sci. 64:1–37.

Babcock, D. F. (1983). Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore. J. Biol. Chem. 258: 6380–6389.

Babcock, D. F., Rufo, G. A. and Lardy, H. A. (1983). Potassium dependent increases in cytosolic pH stimulates metabolism and motility of mammalian sperm. Proc. Natl. Acad. Sci. USA 80: 1327–1331.

Bedford, J. M. (1975). Maturation, transport, and fate of spermatozoa in the epididymis. In: Handbook of Physiology–Endocrinology. Vol. V. American Physiological Society, Washington, D.C., pp. 303–313.

Boron, W. F. and DeWeer, P. (1976). Active proton transport stimulation by CO_2/HCO^{3-}, blocked by cyanide. Nature 259:240–241.

Boron, W. F., McCormick, W. C. and Roos, A. (1979). pH regulation in barnacle muscle fibers: dependance on intracellular and extracellular pH. Am. J. Physiol. 273(Cell physiol.):C185–C193.

Brandt, H., Acott, T. S., Johnson, D. J. and Hoskins, D. D. (1978). Evidence for an epididymal origin of bovine sperm motility protein. Biol. Reprod. 19:830–835.

Cassillas, E. R. (1973). Accumulation of carnitine by bovine spermatozoa during maturation in the epididymis. J. Biol. Chem. 248:8227–8232.

Ellinwood, W. E., Brenner, R. M., Hess, D. L. and Resko, J. A. (1980). Testosterone synthesis in rhesus fetal testis: comparison between middle and late gestation. Biol. Reprod. 22:955–963.

Garbers, D. L., Tubb, D. J. and Hyne, R. V. (1983). Requirement of bicarbonate for Ca^{2+} induced elevations of cyclic AMP in guinea pig spermatozoa. J. Biol. Chem 277:8980–8984.

Goh, P. (1983). Ph.D. Thesis, Sydney University.

Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89:114–123.

Hammerstedt, R. H. and Hay, S. R. (1980). Effect of temperature on motility and cAMP content of bovine sperm. Arch. Biochem. Biophys. 199: 427–437.

Harper, J. F. and Brooker, G. (1975)l Femtomole sensitive radio-immunoassay for cyclic AMP and cyclic GMP after 2′-O acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucl. Res. 1:207–218.

Harrison, R.A.P. (1976). A highly efficient method for

washing mammalian spermatozoa. J. Reprod. Fertil. 48:347–358.

Henle, G. and Zittle, L. A. (1942). Studies of the metabolism of bovine epididymal spermatozoa. Am. J. Physiol. B6:70–78.

Hoskins, D. D., Stephens, D. T. and Hall, M. L. (1974). Cyclic adenosine 3′:5′-monophosphate and protein kinase levels in developing bovine spermatozoa. J. Reprod. Fertil. 37:131–133.

Hoskins, D. D., Hall, M. L. and Munsterman, D. (1975). Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors. Biol. Reprod. 13:168–176.

Hoskins, D. D., Brandt, H. and Acott, T. S. (1978). Initiation of sperm motility in mammalian epididymis. Fed. Proc. 37:2534–2542.

Hoskins, D. D., Johnson, D., Brandt, H. and Acott, T. S. (1979). Evidence for a role for a forward motility protein in the epididymal development of sperm motility. In: The Spermatozoon (D. Fawcett and J. M. Bedford, eds.) Urban and Schwarzenberg, Baltimore, p. 43.

Huang, M. and Daly, J. W. (1974). Adenosine-elicited accumulation of cyclic AMP in brain slices: potentiation by agents which inhibit uptake of adenosine. Life Sci. 14:489–503.

Katz, D. F. and Overstreet, J. W. (1981). Sperm motility assessment by videomicrography. Fert. Steril. 35:188–193.

Lee, H. C., Johnson, C. and Epel, D. (1983). Changes in internal pH of sea-urchin sperm associated with activation. Dev. Biol. 95:31.

Roos, A. and Boron, W. F. (1981). Intracellular pH. Phys. Rev. 61:296–436.

Seamon, K. B. and Daly, J. W. (1981). Forskolin: A unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucl. Res. 7: 201–224.

Steiner, A. L., Parker, C. W. and Kipnis, D. M. (1972). Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. J. Biol. Chem. 247:1106–1113.

Thomas, J. A., Buchsbaum, R. N., Zimniak, A. and Racker, E. (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18:2210–2218.

Triggle, D. J. (1982). Biochemical pharmacology of calcium blockers. In: Calcium Blockers: Mechanism of Action and Clinical Applications (S. F. Flain and R. Zelis, eds.) Urban and Schwarzenberg, Baltimore, pp. 121–134.

Wong, P.Y.D., Lee, W. M. and Tsang, A.Y.F. (1981). The effects of extracellular sodium on acid release and motility initiation in rat cauda epididymal spermatozoa in vitro. Exp. Cell. Res. 131:97–104.

Yanagimachi, R. (1975). Acceleration of the acrosome reaction and activation of guinea pig spermatozoa by detergents and other reagents. Biol. Reprod. 13:519–526.