Effects of Phosphodiesterase Inhibitors and Cyclic Nucleotides on Sperm Respiration and Motility*

David L. Garbers, W. D. Lust,† Neal L. First, and Henry A. Lardy‡

ABSTRACT: Caffeine, theophylline, and papaverine, inhibitors of cyclic nucleotide phosphodiesterase, markedly increased respiration (3- to 4-fold) and motility of bovine epididymal spermatozoa incubated with pyruvate, acetate, oxalacetate, or β-hydroxybutyrate. Respiration in the presence of glucose, fructose, lactate, citrate, α-ketoglutarate, succinate, fumarate, or malate plus caffeine was not as greatly stimulated. N^6,2′-*O*-Dibutyryl cyclic 3′,5′-adenosine monophosphate (diCAMP) and high levels of cyclic 3′,5′-guanosine monophosphate (CGMP) plus 2 mM caffeine stimulated both sperm motility and respiration linked to endogenous substrate utilization. On prolonged incubation, added acetate, pyruvate, or β-hydroxybutyrate was necessary to maintain the respiration elevated by diCAMP whereas no stimulation above the endogenous rate occurred with fructose or α-ketoglutarate as substrates. Cyclic 3′,5′-adenosine monophosphate (CAMP) activated sperm respiration and motility only occasionally, suggesting variations in permeability. The cyclic 3′,5′-nucleotides uridine, cytosine, deoxythymidine, and inosine did not appreciably affect sperm respiration or motility.

Papaverine and caffeine increased CAMP concentrations in sperm incubated with pyruvate. Caffeine without substrate did not appear to increase CAMP concentrations after a 1-hr incubation. CGMP concentrations appeared to be greater than CAMP concentrations, and were increased in sperm incubated with caffeine plus pyruvate compared to pyruvate alone. Both phosphodiesterase inhibitors caused marked decreases in sperm ATP concentrations. Imidazole, at high concentrations, inhibited sperm motility and diCAMP was able to restore tail flagellation, whereas caffeine exerted only slight effects. Caffeine and papaverine initiated progressive motility in ejaculated spermatozoa of poor motility or in aged bovine epididymal sperm. The results suggest an involvement of the nucleotides CGMP and/or CAMP in the control of sperm motility and metabolism.

Cyclic 3′,5′-adenosine monophosphate[1] has been shown to have effects in a wide variety of biological systems (Sutherland *et al.*, 1968). More recently, attention has also been focused on the activity of CGMP (Price *et al.*, 1967). Gray *et al.* (1970) reported the presence of adenyl and guanyl cyclases in sea urchin spermatozoa and levels of $1\text{–}9 \times 10^{-7}$ mmole of cyclic nucleotides per gram wet weight of tissue. An activation of monkey spermatozoan adenyl cyclase by thyroxine in the presence of caffeine has recently been reported (Casillas and Hoskins, 1970). In view of these observations we began an investigation into the possible effects of cyclic nucleotides on bovine spermatozoa. This report presents results which indicate that exogenously added cyclic nucleotides and phosphodiesterase inhibitors have marked effects on the respiration and motility of bovine spermatozoa.

Materials and Methods

Bovine epididymal spermatozoa were collected from the distal caudal epididymis by the method of Henle (1938). The standard collecting medium contained 130 mM KCl, 10 mM KH_2PO_4, 10 mM Tris (Cl), and 5 mM $MgSO_4$, final pH 7.2. Sperm were sedimented at 1000*g* for 5 min, washed once in collecting medium, and finally suspended at a concentration of 1.45×10^8 sperm ml^{-1}. Sperm numbers were read from a standard curve established by relating optical density at 560 nm to sperm counts made with a hemocytometer. In several experiments, 130 mM NaCl or 250 mM sucrose replaced the KCl in the collecting medium, and in others appropriate amounts of KCl or sucrose were deleted to retain isotonic conditions as imidazole was added.

Sperm (10^8 cells) were incubated at 37° in a 1-ml volume and respiration was monitored manometrically using flasks of 7-ml total volume (Morton and Lardy, 1967a). Unless otherwise noted, the incubation medium was the same as the collecting medium described above. Substrates were added at 10 mM final concentration.

Motility ratings were obtained by examination of sperm with a Zeiss phase-contrast microscope at 125× magnification. A grading system based both on per cent tail flagellation and progressiveness of movement was employed. Samples were given a value of 0–6 for an increasingly greater percentage of tail flagellation plus a value of 1 where no progression was observed, 2 for partial progression, and 3 for excellent progression. The sum of the two scores is the motility rating. An investigator unaware of treatment groups rated most samples.

In experiments where low-motility sperm were desired, epididymal sperm were aged for 4 hr at 37° in the presence or absence of substrate. Alternately, ejaculated sperm of known poor quality were obtained from the American Breeders Service, Madison, Wis. Fresh or frozen poor quality sperm

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† From the Department of Pharmacology, University of Minnesota, Minneapolis, Minn.

‡ To whom to address correspondence.

[1] Abbreviations used in the text are: CAMP, cyclic 3′,5′-adenosine monophosphate; CGMP, cyclic 3′,5′-guanosine monophosphate; diCAMP, N^6,2′-*O*-dibutyryl cyclic 3′,5′-adenosine monophosphate; CUMP, cyclic 3′,5′-uridine monophosphate; CIMP, cyclic 3′,5′-inosine monophosphate; CdTMP, cyclic 3′,5′-deoxythymidine monophosphate; CCMP, cyclic 3′,5′-cytidine monophosphate.

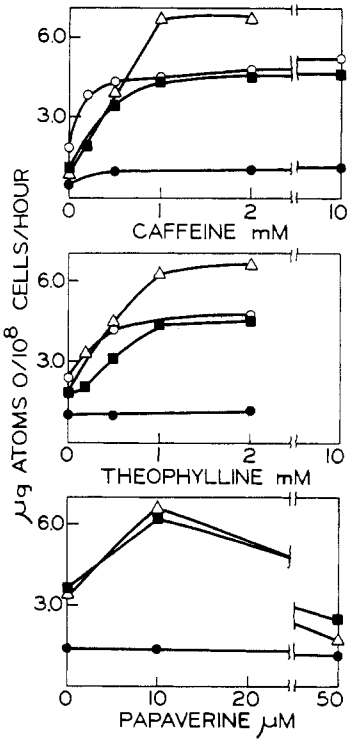

FIGURE 1: The effect of three phosphodiesterase inhibitors on bovine epididymal sperm respiration. Spermatozoa were incubated for 1 hr at 37° in the medium described in the Methods. (—●—) No exogenous substrate, (—△—) 10 mM acetate, (—○—) 10 mM oxalacetate, and (—■—) 10 mM pyruvate.

were centrifuged from the ABS commercial diluent and resuspended in the KCl buffer.

Cyclic nucleotides and ATP were measured in extracts of sperm which had been incubated for 1 hr at 37°. After incubation, sperm were centrifuged at 450*g* for 15 min, the supernatant fluid was decanted, and 1.75 ml of ice-cold 10% trichloroacetic acid was added to the 35.0 mg wet weight of sperm (10^8 cells = 2.2 mg dry weight or 11.6 mg wet weight). Samples were mixed and homogenized on ice with a Potter-Elvehjem homogenizer for 50 or 100 strokes. The sperm were sedimented by centrifugation at 1600*g*, the supernatant fluid was removed, and trichloroacetic acid extracted from this by three extractions with water-saturated ether. Samples were then frozen until assay. Cyclic 3′,5′-AMP and cyclic 3′,5′-GMP were assayed by the method of Goldberg *et al.* (1969a,b) and ATP was measured fluorimetrically (Greengard, 1963).

Bovine epididymides were donated by Oscar Mayer and Co., Madison, Wis. Papaverine was a gift from R. O'Dea. Theophylline and caffeine were obtained from Calbiochem or Eastman Organic Chemicals. Imidazole and the cyclic nucleotides were purchased from Calbiochem or Boehringer-Mannheim. Substrates were added as potassium or sodium salts except for lithium lactate and were the best grade available.

Results

Table I shows the oxygen consumption of bovine epididymal spermatozoa incubated with various substrates in the presence or absence of the phosphodiesterase inhibitor caffeine. Respiration rates in the absence of caffeine agree well with an earlier report (Lardy *et al.*, 1945). Caffeine (2 mM) had relatively little effect on oxygen consumption with no added substrate or that observed in the presence of glucose, fructose, citrate, α-ketoglutarate, succinate, and fumarate. Caffeine produced a mild elevation of oxygen consumption with malate and lactate as substrates and a marked increase in respiration when pyruvate, acetate, β-hydroxybutyrate, or oxalacetate were added. Since the respiratory stimulation appeared greater with these latter substrates, they were employed in subsequent studies.

TABLE I: Respiration of Bovine Epididymal Spermatozoa with Various Substrates at 10 mM in the Presence and Absence of Caffeine.[a]

10 mM Substrate	*n*	μg-atoms of O/10^8 Cells/hr: Substrate Only	+2 mM caffeine	Increase
Endogenous	8	0.80	1.20	0.40
Glucose	1	2.08	2.69	0.61
Fructose	4	1.68	2.88	1.20
Lactate	3	1.18	3.00	1.82
Pyruvate	12	1.48	4.90	3.42
Acetate	7	1.45	4.21	2.76
Citrate	4	0.90	1.31	0.41
α-Ketoglutarate	4	0.91	1.27	0.36
Succinate	1	1.46	1.69	0.23
Fumarate	3	0.82	1.11	0.29
Malate	2	1.12	2.53	1.41
Oxalacetate	3	1.63	4.88	3.25
β-Hydroxybutyrate	2	1.17	4.04	2.87

[a] Sperm were incubated for 1 hr at 37° in the medium described in the Methods. The number of experiments is recorded under *n*.

The stimulation of sperm respiration in the presence of pyruvate, acetate, or oxalacetate as a function of the concentration of two methylxanthines is presented in Figure 1. Caffeine and theophylline exhibited similar dose–response relationships and maximally increased oxygen uptake by sperm at a concentration of 1 mM. The more potent phosphodiesterase inhibitor papaverine (Triner *et al.*, 1970; O'Dea *et al.*, 1970), at a concentration of 0.01 mM, produced a doubling of sperm respiration in the presence of substrate, but like the methylxanthines, it had no effect on sperm oxygen uptake in the absence of added substrate (Figure 1). High concentrations of papaverine proved inhibitory to sperm respiration. The level of respiratory stimulation by caffeine plus substrate equaled or exceeded the increased respiration induced by dinitrophenol (DNP). Levels of 0.01–0.50 mM DNP in the presence of substrate caused a maximal respiratory response of between 5.5 and 6.5 μg-atoms of O/10^8 cells per hr. This is in agreement with stimulations of oxygen consumption by DNP reported earlier (Lardy and Phillips, 1943a).

Since the methylxanthines and papaverine could produce elevated levels of cyclic 3′,5′-nucleotides in sperm by inhibition of phosphodiesterase, the effects of exogenous cyclic nucleotides on bovine sperm respiration were examined. The addition of CUMP, CdTMP, CIMP, and CCMP had no marked effect on sperm cell respiration (Table II). 5′-AMP did not stimulate either respiration or motility in the presence of acetate, pyruvate, or citrate.

TABLE II: The Effect of Various Cyclic Nucleotides on Bovine Epididymal Sperm Respiration.[a]

Addition	μg-atoms of O/10⁸ Cells/hr		
	Endogenous	+10 mM Acetate	+2 mM Caffeine
None	1.21	1.79	2.37
CUMP	2.05	1.65	2.68
CdTMP	1.47	1.47	1.87
CIMP	1.87	1.47	2.99
CGMP	2.50	2.10	6.20
CCMP	1.25	1.12	2.05
CAMP	1.52	2.28	3.30
DiCAMP	6.12	3.17	9.71

[a] All cyclic nucleotides were added to the incubation to a final concentration of 5 mM. Incubation was for 1 hr at 37° with no substrate, no substrate plus 2 mM caffeine, or with acetate at 10 mM.

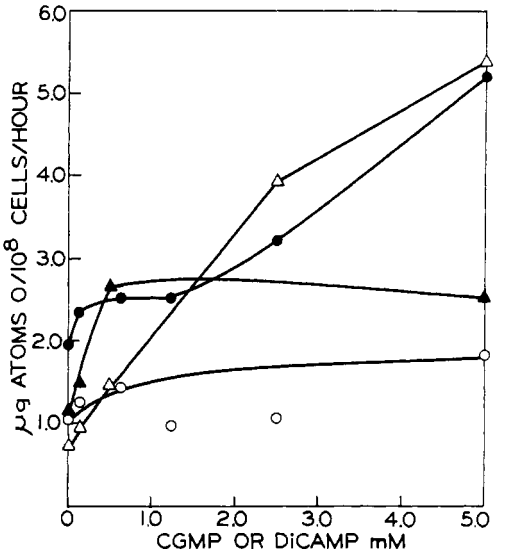

FIGURE 2: The effects of diCAMP and CGMP on bovine epididymal sperm respiration. Spermatozoa were incubated for 1 hr at 37° in the medium described in the Methods. (—○—) CGMP, 10 mM pyruvate; (—▲—) diCAMP, 10 mM pyruvate; (—●—) CGMP, 2 mM caffeine; (—△—) diCAMP, no exogenous substrate.

Although CAMP appears to have no effect in this set of experiments, in two of six experiments, which are not presented in any table, CAMP stimulated respiration in the presence of pyruvate by 400%. Figure 2 shows that diCAMP produced an increased respiration in the presence of pyruvate at concentrations of cyclic nucleotides as low as 0.1 mM with maximal stimulation at 0.5 mM. In the absence of exogenous substrate, diCAMP produced a linear increase in respiration rate over the range 0.5–5 mM cyclic nucleotide. Sperm may use endogenous phospholipid in the absence of an exogenous energy source (Lardy and Phillips, 1941; Hartree and Mann, 1959). With added pyruvate as substrate, diCAMP stimulated respiration to a lesser extent. Cyclic GMP stimulated sperm respiration only slightly in the absence of added substrate but CGMP plus caffeine produced a more marked increase in oxygen consumption.

Because of the stimulation by diCAMP of endogenous respiration rate, the substrate specificity of the diCAMP activation was more difficult to study. With time, however, the endogenous stimulation by diCAMP decreases. After 5-hr incubation, differentiation of substrates involved with the diCAMP activation from those not involved becomes possible. The effects of diCAMP (5 mM) are similar to those observed with caffeine (Table III). In the presence of β-hydroxybutyrate, pyruvate, and acetate, respiration is stimulated by diCAMP over the diCAMP stimulation of endogenous rate. Fructose and α-ketoglutarate do not stimulate respiration over that of diCAMP alone.

Since seminal plasma is the richest source of the prostaglandins (Mann, 1964), and because prostaglandins have been demonstrated to affect CAMP levels in various tissues (Robison *et al.*, 1968), it was of interest to determine if prostaglandins could simulate the effect of caffeine upon sperm. In the presence of pyruvate or glucose, prostaglandins $E_{1\alpha}$, E_1, and E_2, at 20 μg/ml, caused no stimulation of oxygen consumption, and sperm motility was unaffected. Pento *et al.* (1970) had previously reported no stimulation of ram epididymal sperm respiration by prostaglandins in the presence of glucose. Prostaglandin E_1 at levels of 20 or 40 μg per ml caused no detectable change in CAMP levels of spermatozoa incubated for 1 hr with the prostaglandin, 2 mM caffeine, and 10 mM pyruvate.

In view of the finding that exogenous CGMP or diCAMP stimulated sperm respiration, it was of interest to determine whether the elevation in oxygen consumption produced by caffeine plus substrate was associated with a change in the levels of CAMP or CGMP. After a 1-hr incubation with or without pyruvate, bovine sperm contained higher levels of CAMP or CGMP (Figure 3) than were reported for sea urchin sperm (Gray *et al.*, 1970). Caffeine alone did not increase the concentration of CAMP in sperm during a 1 hr incubation whereas low concentrations of caffeine plus pyruvate increased CAMP slightly. A high concentration of caffeine

TABLE III: Respiration of Bovine Epididymal Spermatozoa Incubated with 5 mM DiCAMP or 2 mM Caffeine in the Presence of 10 mM Substrate.[a]

10 mM Substrate	Respiration Expressed as % of Endogenous	
	+2 mM Caffeine	+5 mM DiCAMP
Endogenous	100	100
Fructose	120	78
Pyruvate	235	161
Acetate	230	151
α-Ketoglutarate	90	107
β-Hydroxybutyrate	215	145

[a] Oxygen consumption was measured at 37° for 5 hr in the KCl buffer described in the Methods. The endogenous respiration (μg-atoms of O/10⁸ cells per hr) in the presence of 5 mM diCAMP was 2.27 and in the presence of 2 mM caffeine was 1.20.

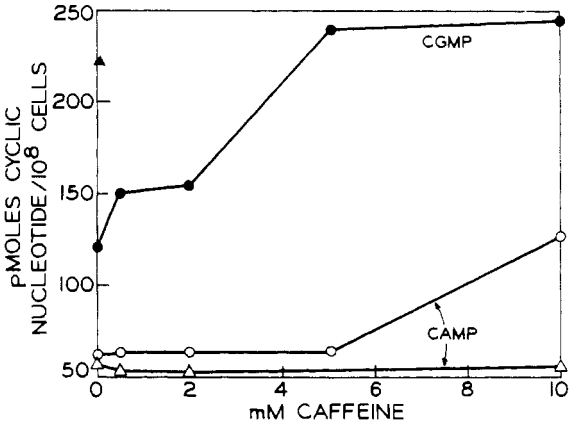

FIGURE 3: The effect of caffeine on CAMP and CGMP concentrations in bovine epididymal spermatozoa. Spermatozoa were incubated for 1 hr at 37° and prepared for assay of CAMP and CGMP as described in the Methods. (—▲—) Level of CGMP in sperm incubated in buffer, without substrate or caffeine present; (—△—) no exogenous substrate; (—○—) 10 mM pyruvate; (—●—) 10 mM pyruvate.

(10 mM) plus pyruvate markedly increased CAMP. The concentration of CGMP was lower in sperm incubated with pyruvate than in sperm incubated in buffer alone. In the presence of pyruvate, caffeine caused an increase in CGMP, and by 5 mM caffeine, CGMP concentrations were greater than those in sperm incubated with only buffer. The effect of caffeine alone on sperm CGMP concentrations is not yet known. Preliminary experiments indicate that papaverine also markedly increases CAMP levels in bovine epididymal sperm.

Both phosphodiesterase inhibitors markedly decreased the amount of ATP in sperm (Table IV).

Since agents that uncouple oxidative phosphorylation stimulate spermatozoan respiration but inhibit sperm motility (Lardy and Phillips, 1943b, 1945), it seemed appropriate to investigate the effects of phosphodiesterase inhibitors and cyclic 3′,5′-nucleotides on sperm motility. Epididymal spermatozoa are motile immediately after collection in the KCl buffer, but their motility decreases during a 1-hr incubation at

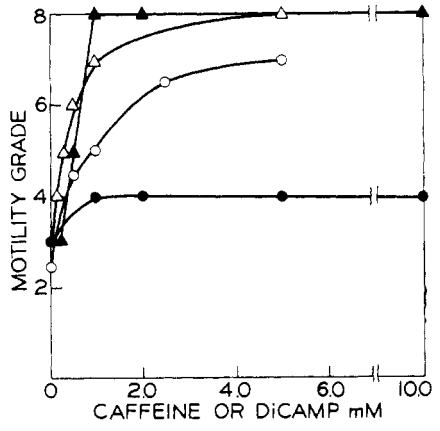

FIGURE 4: The effects of various levels of exogenous caffeine and diCAMP on sperm cell motility. Spermatozoa were incubated for 1 hr in the buffer described in the Methods. Motilities were graded immediately as described in the text. (—●—) Caffeine, no exogenous substrate; (—○—) diCAMP, no exogenous substrate; (—△—) diCAMP, 10 mM pyruvate; (—▲—) caffeine, 10 mM pyruvate.

TABLE IV: Effects of Caffeine and Papaverine on Bovine Epididymal Sperm ATP Levels and Motility.[a]

Addition	10 mM Caffeine	nmoles of ATP/10^8 Cells	Motility
None	—	21.0	2
	+	4.2	4
Pyruvate	—	24.2	3
	+	18.6	7
Acetate	—	20.8	3
	+	6.2	7
Citrate	—	21.1	3
	+	5.3	4
α-Ketoglutarate	—	11.2	3
	+	4.3	4
Fructose	—	18.4	2
	+	17.5	6
0.01 mM papaverine	—	5.4	2
0.01 mM papaverine + pyruvate	—	11.3	6

[a] Sperm were incubated with or without 10 mM substrate for 1 hr at 37° and then extracted for measurement of ATP as described for the CAMP assay in the Methods. Sperm motility was determined after a 4-hr incubation in the presence or absence of 10 mM caffeine at 37° in the KCl buffer.

37° in the presence or absence of substrate. Figure 4 shows that when sperm are incubated in the presence of various concentrations of caffeine plus pyruvate, but not caffeine alone, motility is markedly greater after a 1-hr incubation. Maximal stimulation of motility appears at 1 mM caffeine and the motility is progressive. Sperm incubated with various concentrations of diCAMP plus 10 mM pyruvate also showed markedly enhanced motility and the cyclic nucleotide was able to stimulate motility appreciably in the absence of exogenous substrate. Sperm incubated with various concentrations of CGMP plus 2 mM caffeine also showed marked motility after 1-hr incubation, whereas CGMP alone was ineffective. The stimulation of progressive motility by caffeine plus pyruvate was also observed when sperm were incubated in media in which the KCl solution was replaced with isotonic NaCl or sucrose. Thus the stimulation of motility was not simply due to relief of an inhibition of motility by high concentrations of KCl (Salisbury, 1962).

The presence of certain exogenous substrates was shown to be necessary to allow caffeine to stimulate respiration of sperm. It was of interest, therefore, to determine if a substrate-specific increase in motility of bovine sperm could be demonstrated. Sperm incubated for 4 hr with either pyruvate or acetate plus caffeine showed enhanced motility with excellent progression when compared to sperm incubated with caffeine and no substrate or substrate alone (Table IV). Caffeine plus α-ketoglutarate or citrate was unable to maintain motility to an extent much greater than in the absence of substrate after the 4-hr incubation.

High concentrations of imidazole appear to activate CAMP phosphodiesterase activity (Butcher and Sutherland, 1962; O'Dea *et al.*, 1970; Nakano *et al.*, 1970). When sperm are collected and incubated in buffers containing high levels

TABLE V: The Effects of Caffeine and DiCAMP on Respiration and Motility of Bovine Epididymal Sperm Incubated in Imidazole.[a]

Concn of imidazole / Addition	75 mM		107 mM	
	μg-atoms of O/10⁸ Cells/hr	Motility	μg-atoms of O/10⁸ Cells/hr	Motility
None	1.00	2	0.66	2
10 mM pyruvate	2.94	3	2.46	2
2 mM caffeine	1.05	2		
Caffeine + pyruvate	5.25	4	3.63	3
5 mM diCAMP	5.35	3	3.88	5
DiCAMP + pyruvate	5.91	8	4.13	6

[a] Spermatozoa were incubated for 5 hr at 37° in either 75 or 107 mM imidazole in the presence of the addition.

TABLE VI: Initiation of Motility by Phosphodiesterase Inhibitors and Cyclic Nucleotides in Bovine Spermatozoa.

Expt	Addition	No Substrate	+10 mM Pyruvate
I[a]	None	5 N	5 N
	2 mM caffeine	40 P	65 P
	5 mM diCAMP	10 N	5 N
	5 mM CGMP	10 N	20 N
		Frozen	Fresh
II[b]	None	10 N	10 N
	3.2 mM caffeine	30 P	30 P
	8 mM diCAMP	10 N	10 N
	8 mM CGMP	10 N	10 N
	0.03 mM papaverine	30 P	30 P
III[c]	None	15 N	5 PP
	3.2 mM caffeine	50 P	60 P
	8 mM diCAMP	10 N	25 PP
	8 mM CGMP	10 N	20 PP
	8 mM CAMP	20 N	25 PP

[a] In expt I, sperm were incubated 4 hr at 37° in the KCl medium. Additions were then made and motilities read within 15 min. The number represents the per cent of sperm with tail flagellation, and the letter represents whether the sperm were N, not progressive; PP, partially progressive; or P, progressive. [b] In expt II, poor motility sperm were kept in the American Breeders Service diluent. Additions were made and motilities read 0.5–2 hr after additions. [c] In expt III, sperm of poor quality obtained in ABS diluent were suspended in the KCl medium. The additions were made and motilities read as in expt II.

of imidazole, motility is immediately inhibited (Table V) either in the presence or absence of pyruvate. When sperm are incubated with 2 mM caffeine plus 10 mM pyruvate in the presence of imidazole, respiration is enhanced but motility is only partly restored. DiCAMP (5 mM) with or without pyruvate effectively overcomes the effect of 107 mM imidazole in inhibiting tail flagellation. Respiration was stimulated by diCAMP, but not significantly greater than the stimulation induced by caffeine in the presence of pyruvate.

In view of the effects of caffeine plus pyruvate and diCAMP in maintaining initial motility, experiments were conducted to test whether these treatments were able to initiate progressive motility in immotile sperm. Sperm were incubated for 4 hr at 37° in order to depress motility. The subsequent addition of 2 mM caffeine produced an immediate progressive motility whether added substrate was present or not, whereas the cyclic nucleotides at 5 mM concentration had very slight effects (Table VI, expt I). The immediate induction of motility by caffeine was maintained over longer periods of incubation only in the presence of added substrate. When sperm aged for 4 hr at 37° were incubated with 5 mM diCAMP plus pyruvate for longer periods of time (7–20 hr at 25°), progressive motility was slowly acquired and maintained.

When poor-quality ejaculated spermatozoa with low motility were obtained in a diluent and treated with 3.2 mM caffeine, an immediate progressive motility was initiated (Table VI, expt II). Papaverine at a concentration of 0.03 mM had a similar effect whereas the cyclic nucleotides tested had no apparent effect. Poor-quality sperm resuspended in the KCl medium showed immediate initiation of progressive motility by 3.2 mM caffeine whether sperm were fresh or had been frozen (Table VI, expt III). The cyclic nucleotides tested on fresh, poor-quality sperm showed a partial initiation of motility which was partially progressive (Table VI, expt III).

Discussion

Spermatozoa are known to demonstrate an increased respiratory rate during at least two time periods of their life, these being at ejaculation and upon contact with female fluids (Iritani *et al.*, 1969). Sperm are also known to become motile at ejaculation, and it has been reported that sperm become "vigorously motile" at about the time of capacitation (Yanagimachi, 1969). Recent work indicates increased motility and a different pattern of tail flagellation at about the time of capacitation in Hamster sperm (Yanagimachi, 1970). Stimulation of sperm respiration and motility, therefore, appears to be correlated with an increased "readiness" for the process of penetration and fertilization of the ovum.

The results reported in this paper suggest that CAMP or CGMP may play a role in the regulation of bovine spermatozoan motility and respiration. Although the concentrations of diCAMP or CGMP necessary to elicit responses similar to those of caffeine in sperm are high (5 mM), this may be due to a sperm plasma membrane barrier to cyclic nucleotide penetration. Both methylxanthines and papaverine are known to inhibit phosphodiesterase, an enzyme that hydrolyzes cyclic nucleotides to nucleoside 5′-phosphates. Both types of phosphodiesterase inhibitors markedly stimulate sperm motility and respiration associated with certain exogenous substrates. Furthermore, the relative concentrations of methylxanthines or papaverine that cause these stimulations parallel the reported relative potency of these alkaloids with respect to their inhibition of phosphodiesterase activity (Triner *et al.*, 1970; O'Dea *et al.*, 1970). Casillas and Hoskins (1970) have shown that methylxanthines prevent destruction

of exogenous radioactively labeled CAMP incubated with monkey spermatozoa, suggesting the presence of phosphodiesterase activity in mammalian sperm. High concentrations of imidazole are known to potentiate CAMP phosphodiesterase activity (Butcher and Sutherland, 1962; O'Dea *et al.*, 1970; Nakano *et al.*, 1970), and were shown in our studies to markedly depress motility of bovine epididymal spermatozoa incubated with or without pyruvate and all caffeine-stimulated motility. At these high concentrations imidazole could be acting as a chelating agent, depriving the sperm of essential cations. However, diCAMP was able to overcome the imidazole inhibition of motility, which suggests that imidazole was acting to stimulate destruction of endogenous cyclic nucleotides.

CAMP content of bovine sperm incubated with caffeine and no substrate for 1 hr did not increase. Incubation with caffeine plus pyruvate, however, elevated CAMP concentrations and stimulated respiration and motility. Though the increased CAMP concentrations associated with enhanced respiration and motility were slight, it should be noted that other systems, where CAMP is thought to act as a second messenger, may be maximally stimulated with no change or only small changes in the level of cyclic nucleotides (Namm *et al.*, 1968). CGMP concentrations were lower in sperm incubated with pyruvate than with no substrate, but were increased by increasing levels of caffeine in the presence of pyruvate.

A more direct means of assessing the influence of cyclic 3′,5′-nucleotides on sperm metabolism and motility is the incubation of sperm in the presence of these cyclic nucleotides. The effects of exogenous cyclic nucleotides, however, may be complicated by the low degree of penetrability and rapid hydrolysis of the naturally occurring compounds. DiCAMP has been shown by others to be more effective than CAMP, presumably because of its resistance to hydrolysis by phosphodiesterase and its greater lipid solubility (Robison *et al.*, 1968). Experiments with radioactive labeled cyclic nucleotides and caffeine have demonstrated that CGMP and caffeine penetrate the sperm cell membrane whereas CAMP does not. No information on the penetrability of diCAMP is yet available. The divergent effects of diCAMP and CAMP on the isolated fat cell, however, stress caution in assuming that these two nucleotides act alike in all cases (Solomon *et al.*, 1970). The N^6,2′-*O*-dibutyryl derivative of cyclic AMP caused a large stimulation of bovine spermatozoan respiration either in the presence or absence of exogenous substrate and likewise markedly increased motility with or without pyruvate. With a longer incubation time, diCAMP exhibited a substrate specificity with regard to the stimulation of respiration similar to that of caffeine. CAMP was inconsistent in its effects on spermatozoa while high levels of CGMP produced effects similar to diCAMP only in the presence of caffeine. Cyclic nucleotides other than diCAMP and CGMP appeared to have less or no effect on sperm respiration or motility.

ATP, the energy source for sperm motility (Mann, 1964; Morton and Lardy, 1967b), is present in diminished concentrations in caffeine-treated cells which suggests that the ATP flux is greater. Increased ATP utilization could simulate the response caused by dinitrophenol, where the ATP:ADP ratio acts as a regulator of respiratory rate (Lardy and Wellman, 1953).

It should be noted that in procaryotic cells, substrate rather than hormone can act as an activator or inhibitor of adenyl cyclase (Hirata and Hayaishi, 1965; Makman and Sutherland, 1965). It has been shown that partially purified adenyl cyclase from *Brevibacterium liquefaciens* requires pyruvate as a catalytic activator. Oxalacetate and α-ketobutyrate can partially replace pyruvate, but acetate cannot act as an activator (Hirata and Hayaishi, 1965). The relationship of substrate oxidation in sperm to increased motility is under further investigation at present.

Sperm have been shown to contain a "feeble" actomyosin-like protein (Pautard, 1962), and a calcium- and magnesium-activated ATPase has been measured in sperm tails (Voglmayer *et al.*, 1969). Caffeine causes contraction and increased respiration of muscle (Manchester, 1970), and has been shown to produce calcium efflux from the isolated sarcoplasmic reticulum (Fairhurst and Hasselbach, 1970). Increased respiration of toad bladder can be induced by both vasopressin and CAMP (Parisi and Bentley, 1970). The inotropic response of cardiac muscle to catecholamines appears to be mediated by CAMP (Robison *et al.*, 1968). Doses of theophylline sufficient to cause relaxation of smooth muscle to the guinea pig ileum produce an increase in CAMP levels (cited by Robison *et al.*, 1968). Responses of several diverse biological systems to cyclic nucleotides appear to involve calcium (Rasmussen, 1970). The possibility that caffeine and cyclic nucleotides are affecting spermatozoa motility in a manner analogous to their effects on muscle and other tissues is being investigated.

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Autoxidation of Reduced Pyridine Coenzymes and of Their Models Promoted by *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine*

E. J. H. Bechara† and G. Cilento‡

ABSTRACT: In the presence of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine as a free-radical source, the reduced pyridine coenzymes and their models autoxidize by a free-radical mechanism (HO_2 chain). The main products are the pyridinium form of the substrate and hydrogen peroxide. In accord with the HO_2 chain process are: (i) the first-order dependence of the rate on the dihydronicotinamide concentration and the half-order dependence upon both the oxygen and catalyst concentration, (ii) the value 2.3 for the k_H/k_D ratio on substituting hydrogen by deuterium at C_4 of the dihydronicotinamide, (iii) the identical rate in D_2O and H_2O, (iv) the value of 10.0 kcal/mole for the activation energy, and (v) the pH behavior. The work supports the view that biochemical generation of perhydroxyl radicals depends on low redox potentials rather than on specific enzyme structure.

Tetramethyl-*p*-phenylenediamine,[1] presumably in the monoprotonated form, catalyzes the autoxidation of *p*-phenylenediamine and almost certainly its own autoxidation as well (Cilento and Zinner, 1967). TMPD has now been found to catalyze the autoxidation of reduced pyridine coenzymes and of their models.

Since TMPD is commonly used in studies of the respiration–phosphorylation chain this catalyzed autoxidation of 1,4-dihydronicotinamides has been thoroughly investigated.

Materials

1-Benzyl-1,4-dihydronicotinamide (mp 123°) was prepared according to Mauzerall and Westheimer (1955); to prepare the C-4 monodeuterio analog, D_2O was substituted for H_2O in the reaction mixture. 1-*n*-Propyl-1,4-dihydronicotinamide, prepared according to Suelter and Metzler (1960), was recrystallized from water (mp 195°). The reduced pyridine coenzymes and catalase were from Sigma Chemical Co., the alcohol dehydrogenase from C. F. Bohringer und Soehne.

TMPD·2HCl (BDH reagent) was purified by dissolving in absolute methanol; by cooling the saturated solution

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† Predoctoral fellow of the Fundação de Amparo à Pesquisa do Estado de São Paulo.
‡ To whom to address correspondence.

[1] Abbreviation used is: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.