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Control of Ram Sperm Adenylate Cyclase by Divalent Cations

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Abstract

The adenylate cyclase activity of ram sperm increased on freeze–thawing and the enzyme was stable at 0°C. Its activity was stimulated by Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+} in descending order of activity. The enzyme was insensitive to fluoride when Mn^{2+} concentration was in excess. Mn^{2+}-stimulated enzyme activity was decreased by the simultaneous addition of Co^{2+}, or Cd^{2+}, or Ni^{2+}, and particularly Cu^{2+}. Sulfhydryl compounds (viz. dithiothreitol, glutathione, dithiocarbamate, 2-mercaptoethanol, ergothioneine and cysteine) and chelating agents (viz. D-penicillamine and 8-hydroxyquinoline) were effective, to varying degrees, in overcoming the inhibition by Cu^{2+}. Ca^{2+} augmented the stimulatory effect of Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} on enzyme activity.

Introduction

Adenylate cyclase (EC 4.6.1.1, ATP ⇄ 3′,5′-cAMP + pyrophosphate) activity has been measured in the sperm of several species, e.g. monkey (Casillas and Hoskins 1970), human, dog and sea urchin (Gray *et al.* 1971), bull and boar (Garbers *et al.* 1971, 1973*a*, 1973*b*), hamster (Morton and Albagli 1973), rat (Braun and Dods 1975), ram (Towns and Luke 1976), guinea pig (Hyne and Garbers 1979), goat and buffalo (Chaudhry and Anand 1975). The present evidence indicates that adenylate cyclase, through increased levels of cyclic AMP (cAMP), regulates sperm metabolism and motility (Garbers *et al.* 1973*a*) and other components of a 'second-messenger' system have been identified in sperm, including phosphodiesterase (Stephens *et al.* 1979), cAMP-dependent protein kinase (Hoskins *et al.* 1972; Garbers *et al.* 1973*c*) and phosphoprotein phosphatase (Tang and Hoskins 1975).

The reports by Casillas and Hoskins (1970, 1971) Morton and Albagli (1973) and Braun (1975) for monkey, hamster and bull sperm, respectively, indicate that sperm adenylate cyclase has unusual properties compared with those of the enzyme from other tissues. The sperm enzyme is catalytically more active in the presence of Mn^{2+} than with Mg^{2+}, and relatively high concentrations of either metal are required for maximum activity. Furthermore, fluoride ion does not stimulate sperm adenylate cyclase, and no hormonal activator has been identified. Other cations, including Ca^{2+}, Co^{2+} and Zn^{2+}, also support adenylate cyclase activity and Ca^{2+} acts synergistically with Mn^{2+} to reduce the concentration required for maximum activity.

Hitherto, the enzymatic properties of adenylate cyclase in ram sperm have not been characterized in detail especially with regard to metal ion requirements and since divalent cations, as well as cAMP, have been implicated in the mechanism of mammalian sperm motility (White and Holland 1977), this study focuses on the regulation of ram sperm adenylate cyclase by metal ions.

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Materials and Methods

Collection and Preparation of Sperm

Ram semen was collected from Merino rams by electroejaculation (Blackshaw 1954). Samples with good initial motility were pooled, diluted with an equal volume of Ca^{2+}-free Krebs–Ringer phosphate buffer pH 7·4 (Umbreit *et al.* 1972) and the sperm, cytoplasmic droplets and seminal plasma separated by the method of Tash (1976). Ten millilitres of 10% and 5% Ficoll (Pharmacia) solution in HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] buffer (20 mM HEPES, 30 mM NaCl, 5 mM KOH, 2·5 mM Na_2HPO_4, 1 mM $MgSO_4$ and 10 mM glucose, pH 7·2, osmolarity adjusted to 308 mosm l^{-1} with sucrose) were layered in a 30 ml Corex tube. Diluted semen (4 ml) was carefully placed on top of the Ficoll solutions and centrifuged at 20°C in a Sorvall RC-3 centrifuge for 5 min at 600 g and then for 15 min at 1500 g. The top layer and top interface containing the seminal plasma and cytoplasmic droplets were removed. The Ficoll solution that remained in the original Corex tube was discarded and the pelletted sperm were resuspended in ice-cold 50 mM Tris–HCl buffer, pH 7·4, containing 16 mM caffeine and 0·1% bovine serum albumin (TCB buffer, pH 7·4). Sperm in polypropylene tubes were then snap-frozen in liquid nitrogen, rapidly thawed at 30°C and kept at 4°C until assayed for adenylate cyclase activity.

Adenylate Cyclase Activity Measurement

Enzyme activity of freeze–thawed sperm was assayed by measuring the rate of cAMP formation from ATP. Unless otherwise indicated, the assay medium contained 2 mM ATP, 5 mM $MnCl_2 . 4H_2O$ and an ATP-regenerating system consisting of 10 mM phosphocreatine and 1 mg ml^{-1} of creatine phosphokinase (Braun 1975). After preincubating at 37°C the reaction was initiated by adding freeze–thawed sperm suspension; after 10 min the reaction was terminated by adding 0·5 ml of 20 mM EDTA in 50 mM Tris–HCl buffer, pH 7·4, and boiling for 3 min. The addition of EDTA before boiling prevents non-enzymatic formation of cAMP in the presence of Mn^{2+} (Gray 1971). The cAMP produced was determined by a modification of the competitive protein-binding procedure of Brown *et al.* (1970) employing a binding protein from bovine adrenal cortex as the saturable reagent and charcoal to effect separation of bound and free. The validity of the assay was established by critically examining its accuracy, precision, sensitivity and specificity.

Each experiment was replicated on three (n = 3) different sperm samples collected on each occasion from a group of 12 rams, and the data analysed by t-tests.

Results

Effect of Freeze–Thawing Sperm

Since intact cells are impermeable to ATP (Roll *et al.* 1956) and since in many tissues adenylate cyclase is associated with the inside of the plasma membrane (Oye and Sutherland 1966), the adenylate cyclase activity of cell preparations is likely to vary with their degree of disruption. This was checked by comparing the adenylate cyclase activities of untreated and freeze–thawed ram sperm, which were 12·4 ± 0·9 (s.e.) and 23·8 ± 1·5 nmol cAMP/10^9 sperm/min respectively (n = 3, $P < 0·01$ on t-test).

Stability of Ram Sperm Adenylate Cyclase

When suspensions of freeze–thawed ram sperm were kept at 4°C and assayed at intervals in the presence of 5 mM Mn^{2+} in a buffer of pH 7·0 the enzyme activity was fairly stable for at least 8 h. Thus the mean value at zero time for another three sperm samples from different rams was 13·5 ± 0·1 (s.e.) and at 8 h 11·6 ± 0·4 nmol cAMP/10^9 sperm/min ($P < 0·05$).

Dependence of Adenylate Cyclase Activity on Sperm Concentration and Reaction Time

The reaction rate was proportional to the sperm concentration up to at least 1×10^8 sperm per ml of incubation mixture; thus 10, 21, 43 and 66 nmol cAMP were formed over 10 min by aliquots of sperm suspensions containing 0·28, 0·56, 1·12 and $2·24 \times 10^8$ cells in 1 ml of incubation medium (n = 3).

The formation of cAMP was linear over 15 min; thus 20, 41, 62, 75 and 100 nmol cAMP were formed by a sperm suspension after incubation for 5, 10, 15, 20 and 30 min respectively ($n = 3$).

Adenylate Cyclase Activity as a Function of ATP–Metal Concentration

In the presence of Mn^{2+}, the rate of cAMP formation increased with increasing ATP concentrations from 0·125 mM to 2 mM and then levelled from 2 mM to 4 mM. Adenylate cyclase activity, when determined as a function of ATP concentration (0·05–5 mM) at two concentrations of Mn^{2+} (5 mM and 20 mM), gave K_m values of 1·00–1·33 mM calculated from double reciprocal Lineweaver–Burk plots (Fig. 1). The presence of 10 mM fluoride in the assay system decreased the V_{max} and K_m when the fluoride concentration exceeded that of Mn^{2+} (5 mM).

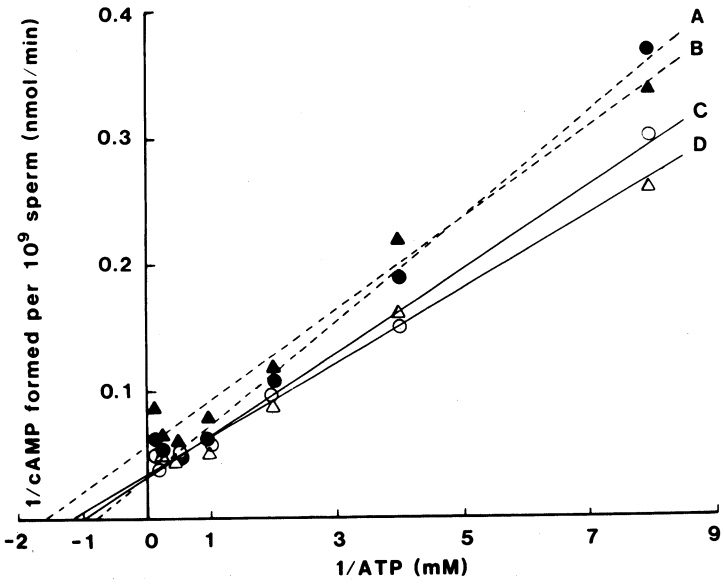

Fig. 1. Lineweaver–Burk double reciprocal plots showing the effect of ATP, Mn^{2+} and F^- concentration on the adenylate cyclase activity of washed and freeze–thawed ram sperm: *A*, 5 mM Mn^{2+}; *B*, 5 mM Mn^{2+} plus 10 mM F^-; *C*, 20 mM Mn^{2+}; *D*, 20 mM Mn^{2+} plus 10 mM F^- ($n = 3$).

Effect of Divalent Cations on Adenylate Cyclase Activity

In the absence of divalent cations, ram sperm adenylate cyclase showed little or no activity. Activity was greatly increased by 5 mM Mn^{2+} to a mean of 19·7 nmol cAMP/10^9 sperm/min, $n = 3$. Zn^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+} were less effective and the mean activity values were 1·6, 1·1, 0·2 and 0·1 respectively.

Antagonism Between Mn^{2+} and Co^{2+}, Cd^{2+}, Ni^{2+} or Cu^{2+} in Stimulating Adenylate Cyclase Activity

The simultaneous addition of 1 mM Mg^{2+} or Co^{2+} to the assay system did not significantly change enzyme activity in the presence of 5 mM Mn^{2+}, thus mean values were 17·7 ± 2·3 and 16·9 ± 0·1 nmol cAMP/10^9 sperm/min (mean ± s.e., $n = 3$) respectively compared with a control value of 18·9 ± 1·1.

However, 1 mM Cd^{2+}, Ni^{2+} or Cu^{2+} reduced the Mn^{2+}-stimulated activity to significantly lower ($P < 0{\cdot}001$) values of $6{\cdot}1 \pm 0{\cdot}3$, $7{\cdot}7 \pm 0{\cdot}5$ and $0{\cdot}9 \pm 0{\cdot}1$ respectively. Cu^{2+} was the most potent inhibitor, 50% inhibition being attained at 0·05 mM Cu^{2+} (Fig. 2).

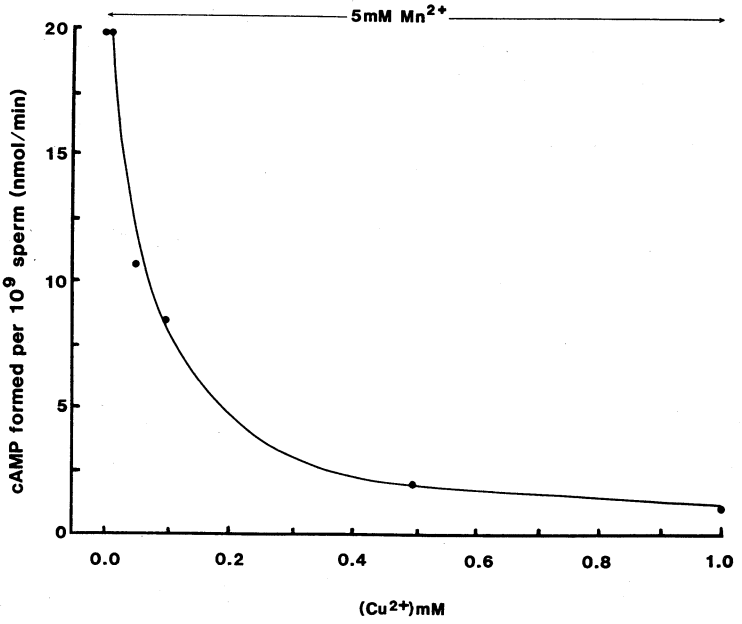

Fig. 2. Inhibition of Mn^{2+}-stimulated adenylate cyclase activity of washed and freeze–thawed ram sperm by Cu^{2+} ($n = 3$).

Table 1. Effect of sulfhydryl compounds and chelating agents on the antagonistic effect of Cu^{2+} on Mn^{2+}-stimulated ram sperm adenylate cyclase

Values are mean ± s.e. ($n = 3$) expressed in nmol cAMP/10^9 sperm/min. * Significantly greater than control with Cu^{2+}, $P < 0{\cdot}05$; ** significantly greater than control with Cu^{2+}, $P < 0{\cdot}01$; *** significantly greater than control with Cu^{2+}, $P < 0{\cdot}001$; † significantly different from value with Cu^{2+} in same row, $P < 0{\cdot}05$; †† significantly different from value with Cu^{2+} in same row, $P < 0{\cdot}01$; ††† significantly different from value with Cu^{2+} in same row, $P < 0{\cdot}001$

Substance added	Adenylate cyclase activity	
	5 mM Mn^{2+}	5 mM Mn^{2+} plus 0·2 mM Cu^{2+}
Control	$16{\cdot}9 \pm 0{\cdot}9^{\dagger}$	$3{\cdot}4 \pm 0{\cdot}3$
2 mM Cleland's reagent	$17{\cdot}5 \pm 0{\cdot}4$	$16{\cdot}8 \pm 0{\cdot}6^{***}$
2 mM glutathione	$17{\cdot}0 \pm 1{\cdot}2$	$28{\cdot}4 \pm 0{\cdot}7^{***}$
2 mM diethyldithiocarbamate	$20{\cdot}0 \pm 1{\cdot}3$	$20{\cdot}2 \pm 0{\cdot}3^{***}$
2 mM D-penicillamine	$14{\cdot}0 \pm 0{\cdot}4$	$14{\cdot}6 \pm 1{\cdot}6^{**}$
0·5 mM 2-mercaptoethanol	$14{\cdot}6 \pm 0{\cdot}7$	$12{\cdot}5 \pm 0{\cdot}4^{***}$
1 mM 2-mercaptoethanol	$11{\cdot}9 \pm 0{\cdot}7$	$13{\cdot}6 \pm 1{\cdot}0^{***}$
2 mM 2-mercaptoethanol	$18{\cdot}9 \pm 0{\cdot}4$	$14{\cdot}3 \pm 1{\cdot}7^{**}$
2 mM L-cysteine	$17{\cdot}6 \pm 1{\cdot}2^{\dagger\dagger}$	$8{\cdot}8 \pm 0{\cdot}9^{**}$
2 mM EDTA	$8{\cdot}0 \pm 0{\cdot}0^{\dagger\dagger\dagger}$	$10{\cdot}9 \pm 0{\cdot}1^{***}$
2 mM ergothioneine	$17{\cdot}4 \pm 1{\cdot}2^{\dagger\dagger}$	$9{\cdot}9 \pm 0{\cdot}7^{**}$
2 mM 8-hydroxyquinoline	$12{\cdot}2 \pm 0{\cdot}2^{\dagger\dagger\dagger}$	$5{\cdot}4 \pm 0{\cdot}2^{*}$

Prevention by Sulfhydryl and Chelating Agents of the Antagonistic Effect of Cu^{2+} on Mn^{2+}-stimulated Adenylate Cyclase Activity

Table 1 shows that inhibition of the Mn^{2+}-stimulated adenylate cyclase activity by Cu^{2+} was prevented to a great extent by the simultaneous addition of 2 mM Cleland's reagent (dithiothreitol, reduced), reduced glutathione (G–SH), diethyldithiocarbamate, D-penicillamine (2 mM) and 2-mercaptoethanol (0·5–2 mM). Approximately 50% of the Mn^{2+}-stimulated activity could also be restored by the simultaneous addition of 2 mM L-cysteine, EDTA or L-ergothioneine; 8-hydroxyquinoline had little restorative effect.

Table 2. Augmentation by Ca^{2+} of the stimulating effect of Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} on ram sperm adenylate cyclase
Values are mean ± s.e. (n = 3) expressed in nmol cAMP/10^9 sperm/min. ** Highly significant greater than no calcium, $P < 0.01$

Divalent cation	Adenylate cyclase activity	
	No Ca^{2+}	Ca^{2+} (5 mM)
None	–	0·05 ± 0·01
0·5 mM Mn^{2+}	0·18 ± 0·02	1·73 ± 0·22**
5 mM Mg^{2+}	0·25 ± 0·01	0·38 ± 0·01**
5 mM Co^{2+}	1·14 ± 0·01	2·61 ± 0·07**
0·5 mM Zn^{2+}	0·20 ± 0·02	0·83 ± 0·04**

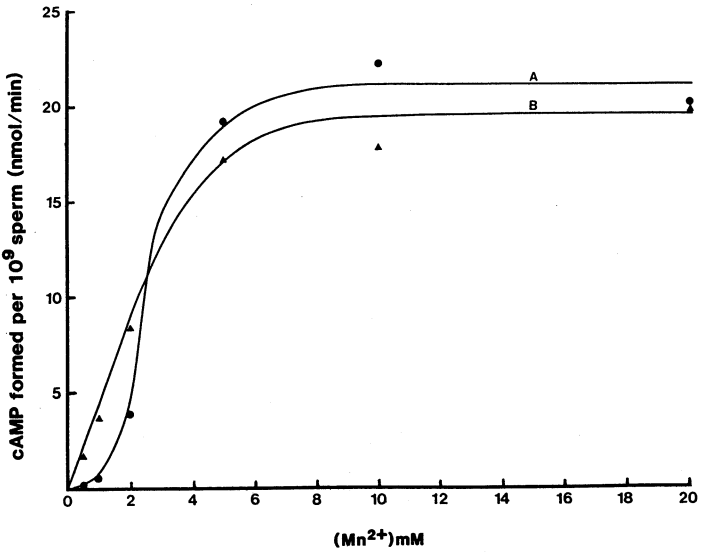

Fig. 3. Potentiation of the stimulatory effect of Mn^{2+} on adenylate cyclase activity of washed and freeze–thawed ram sperm by Ca^{2+}: *A*, Mn^{2+} alone; *B*, Mn^{2+} plus 5 mM Ca^{2+} (n = 3).

Potentiation of the Stimulatory Effect of Mn^{2+}, Mg^{2+}, Co^{2+} or Zn^{2+} on Adenylate Cyclase by Ca^{2+}

Ca^{2+} (2 mM) augmented the stimulatory effect of Mn^{2+} (0·5 mM), Mg^{2+} (5 mM), Co^{2+} (5 mM) and Zn^{2+} (0·5 mM) on adenylate cyclase activity in the presence of 2 mM ATP (Table 2). From Fig. 3, it can be seen that Ca^{2+} augmented the stimulatory effect of only subsaturating concentrations of Mn^{2+}. It was also noted that Ca^{2+} had a potentiating

effect only when the Zn^{2+} concentration in the system was less than that of ATP (2 mM). When the Zn^{2+} concentration was equal to or greater than that of ATP, Ca^{2+} was in fact inhibitory (Fig. 4).

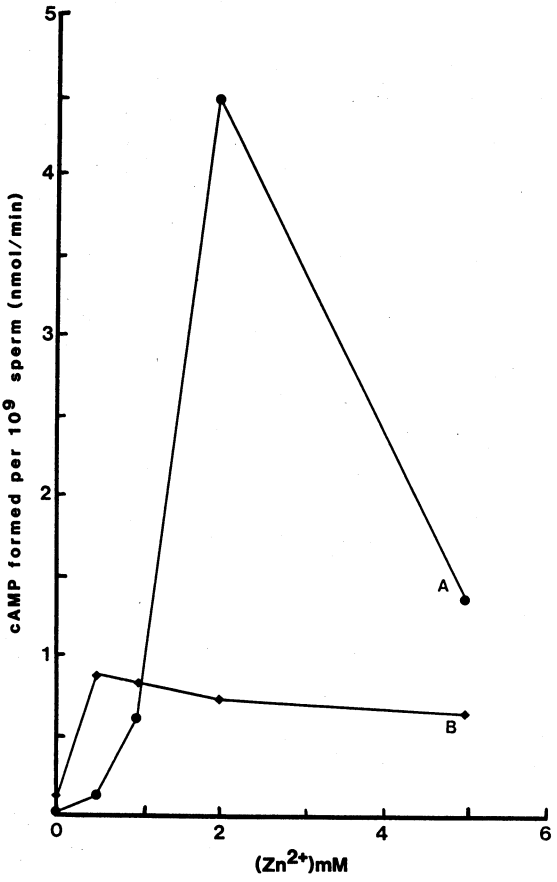

Fig. 4. Effect of Ca^{2+} on adenylate cyclase activity from washed and freeze-thawed ram sperm in the presence of varying concentrations of: *A*, Zn^{2+} alone; *B*, Zn^{2+} plus 5 mM Ca^{2+} (n = 3).

Discussion

It is clear that ram sperm adenylate cyclase is fairly stable when stored at 4°C and resembles bull sperm adenylate cyclase in this respect (Braun 1975). Thus, although the decrease in activity over 8 h was statistically significant, this was only a 14% loss. The higher adenylate cyclase activity of ram sperm following freeze–thawing is most probably due to increased permeability to the substrate (Mann and Lutwak-Mann 1955) and Towns and Luke (1976) have reported an even greater (five-fold) increase.

In general, our observations on the effect of divalent cations and fluoride on ram sperm adenylate cyclase activity concur with those of other workers using monkey (Casillas and Hoskins 1970, 1971), hamster (Morton and Albagli 1973) and bull (Braun 1975) sperm. However, at a Mn^{2+} concentration of 5 mM, the activity was inhibited by 10 mM fluoride, a finding inexplicably at variance with previous studies. K_m values of 1·00–1·33 mM for ATP obtained in the present study were similar to those reported by Hammerstedt and Hay (1980) for bull sperm adenylate cyclase, although Herman *et al.* (1976) found that plasma-membrane-bound adenylate cyclase from bull sperm had a K_m for ATP of approximately 4 mM in the presence of excess Mg^{2+}.

In ram sperm, as in other somatic cells, the substrate for adenylate cyclase is the complex of ATP with a divalent cation. Since Zn^{2+} stimulated the enzyme and is a normal constituent of ram sperm and seminal plasma (Mann 1964), Zn–ATP may be a physiological substrate for the enzyme in these sperm.

The marked Mn^{2+} stimulation of adenylate cyclase activity observed in the present study has also been demonstrated in human, dog (Gray *et al.* 1971), bull (Braun 1975), rat (Braun and Dods 1975) and monkey (Casillas and Hoskins 1971) sperm. However, the concentrations of Mn^{2+} required are far in excess of physiological levels in semen (Mann 1964).

The degree to which the Mn^{2+}-stimulated ram sperm adenylate cyclase activity was inhibited by other divalent cations is in agreement with the order of preference of metals for bidentate nitrogen–sulfur ligands such as cysteine in membrane proteins (Vallee *et al.* 1961). Therefore it may be assumed that, in this case, thiol groups have greater avidity for Co^{2+}, Cd^{2+}, Ni^{2+} or Cu^{2+} than for Mn^{2+}, resulting in a much lower enzyme activity.

The antagonistic effect of Cu^{2+} on Mn^{2+} stimulation of ram sperm adenylate cyclase was completely prevented by the simultaneous addition of Cleland's reagent (dithiothreitol)—a protector of –SH groups (Cleland 1964)—diethyldithiocarbamate or glutathione, which is present in ram sperm and seminal plasma (Li 1975). L-cysteine and L-ergothioneine were much less effective than glutathione in overcoming inhibition of Mn^{2+}-stimulated adenylate cyclase activity by Cu^{2+} but the ability of these sulfhydryl compounds and also D-penicillamine (Bell 1977) and 2-mercaptoethanol to counteract the inhibitory action of Cu^{2+} on Mn^{2+}-stimulated enzyme activity suggests that the binding site for Cu^{2+} is a thiol group, and that the enzyme requires SH-groups in addition to metal ions for activity.

Previous investigations have demonstrated the importance of SH-groups for the motility and metabolism of sperm (Mann 1964) and cysteine and glutathione protect the motility and glycolysis of human, boar and ram sperm from inhibition by Cu^{2+} (MacLeod 1951; Mann and Leone 1953; White 1955).

The chelating agent 8-hydroxyquinoline prevents labilization of the rat liver lysosomal membanes by copper probably by chelating the metal ion (Chvapil *et al.* 1972). However, in the present study, 8-hydroxyquinoline was the least effective agent protecting adenylate cyclase from inactivation by copper. This is perhaps not surprising since White (1956) found that it had spermicidal properties that were enhanced in the presence of copper.

A rather intriguing finding in the present study was that Ca^{2+}, at a concentration of 5 mM, acted synergistically with Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} in stimulating ram sperm adenylate cyclase activity. This effect has also been observed in bull, rat and guinea pig sperm (Braun 1975; Hyne and Garbers 1979).

Our results indicate that in ram sperm, as in somatic cells, divalent cations play a significant role in the regulation of adenylate cyclase activity and it is possible that Ca^{2+}, in concert with other divalent cations, might be a regulator of sperm enzyme activity although the mode of regulation is not known.

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