Influence of 2-deoxy-D-glucose and energy substrates on guinea-pig sperm capacitation and acrosome reaction

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Summary. When guinea-pig spermatozoa were suspended in a minimal culture medium (MCM-PL), 2-deoxy-D-glucose (100 μM) and 2-amino-2-deoxy-D-glucose (100 μM) were potent inhibitors of the acrosome reaction without affecting the sperm motility, whereas the *N*-acetyl derivative 2-acetamido-2-deoxy-D-glucose (2 mM) had no inhibitory effect. The addition of D-glucose (2 mM) partly inhibited the percentage acrosome reaction of spermatozoa suspended in Medium MCM-PL, but DL- α -glycerophosphate (2 mM) and *myo*-inositol (2 mM) had no effect. In addition, DL- α -glycerophosphate (2 mM) did not overcome the inhibitory effect of 2-deoxy-D-glucose on the sperm acrosome reaction. The inhibitory action of 2-deoxy-D-glucose (100 μM) on the sperm acrosome reaction assessed after a 3-h incubation was irreversible and was only completely effective if the sugar was added within 30 min of the start of incubation. When spermatozoa suspended in Medium MCM-PL were treated with 2-deoxy-D-glucose (100–200 μM) for an extended incubation up to 6 h, the inhibitory effect of 2-deoxy-D-glucose was partly overcome. Spermatozoa treated with 2-deoxy-D-glucose had significantly reduced concentrations of ATP after incubation for 2 h in Ca^{2+} -free media, compared with the ATP concentrations of spermatozoa preincubated for 2 h in Ca^{2+} -free media that supported acrosome reactions. The addition of Ca^{2+} (5 mM) caused a rapid decrease in ATP concentrations of spermatozoa suspended in Medium MCM-PL, while the addition of the monovalent ionophore monensin (50 μM) and Ca^{2+} stimulated sperm acrosome reactions as well as an additional decline in the sperm ATP concentrations. However, monensin (50 μM) in the absence of Ca^{2+} caused only a slight decline in the sperm ATP concentrations over the 15-min incubation period. The depletion of the sperm ATP concentrations by 2-deoxy-D-glucose may retard completion of the capacitation process and the resultant acrosome reaction.

Introduction

Sperm capacitation and the acrosome reaction are prerequisites for mammalian fertilization *in vitro* and *in vivo*. Evidence to date suggests that neither the oviduct, the cells and matrix of the cumulus oophorus, the zona pellucida and other products of ovulation nor the oocyte itself are in any way essential for the onset of a normal acrosome reaction in mammalian spermatozoa (Saling & Bedford, 1981; Bedford, 1983). However, organic factors such as serum albumin and other 'serum factors' are essential additives to chemically defined media that permit spermatozoa to undergo capacitation and fertilization *in vitro* (mouse: Hoppe & Whitten, 1974; rat: Niwa & Chang, 1975; Davis, 1976; guinea-pig: Yanagimachi, 1972; Hyne & Garbers, 1981; golden hamster: Lui, Cornett & Meizel, 1977; Mrsny, Waxman & Meizel, 1979; Bavister, 1981; rabbit: Oliphant, 1976; dog: Mahi & Yanagimachi, 1976, 1978; man: Yanagimachi, Yanagimachi & Rogers, 1976; Lopata,

McMaster, McBain & Johnston, 1978). It has long been proposed that capacitation involves endogenous cellular changes that stem primarily from modifications of the plasmalemma of the spermatozoon (Barros, 1974; O'Rand, 1979; Oliphant & Singhas, 1979; Bearer & Friend, 1982), the state of which in turn may regulate a series of biochemical events associated with Ca^{2+} influx (Hyne & Garbers, 1979a, b; Hyne, Higginson, Kohlman & Lopata, 1984).

In addition to the requirement for serum albumin and/or other organic serum factors in sperm capacitating media (Mrsny *et al.*, 1979; Hyne & Garbers, 1981; Bavister, 1981; Quinn, Stanger & Whittingham, 1982), all media also contain exogenous energy-producing metabolites for optimal sperm capacitation and fertilization *in vitro* (Rogers, 1978). Glucose and pyruvate are essential for fertilization of rat eggs (Tsunoda & Chang, 1975), pyruvate plus lactate in the presence or absence of glucose, as well as glucose alone supported acrosome reactions in guinea-pig spermatozoa (Rogers & Yanagimachi, 1975), hamster spermatozoa require exogenous glucose and lactate for acrosome reactions *in vitro* (Dravland & Meizel, 1981), mouse spermatozoa only require glucose for the sperm acrosome reaction and fertilization (Hoppe, 1976; Fraser & Quinn, 1981) and human spermatozoa require glucose for optimal penetration of zona-free hamster eggs or human zonae pellucidae (Perreault & Rogers, 1981; Hoshi, Saito, Suzuki, Hayashi & Yanagimachi, 1982). Media with an elevated pH can overcome the requirement for serum factors to support the acrosome reaction in guinea-pig spermatozoa (Hyne & Garbers, 1981) and possibly dog spermatozoa (Mahi & Yanagimachi, 1978).

The ability of various minimal culture media having an elevated pH to support guinea-pig sperm acrosome reactions is related to culture conditions that increase sperm intracellular Na^+ concentrations (Hyne *et al.*, 1984). Under these culture conditions, the guinea-pig sperm acrosome reaction is supported by glucose in the presence or absence of pyruvate and lactate, but the appearance of the acrosome reaction is delayed compared with control spermatozoa incubated with only pyruvate and lactate. The retarding effect of glucose on the guinea-pig sperm acrosome reaction appears to be related to its metabolism, since other non-metabolizing sugars such as L-glucose and D-fructose did not have a retarding effect (Rogers & Yanagimachi, 1975). In this present study, we investigated the effects of various exogenous carbohydrate metabolites on guinea-pig sperm capacitation and the acrosome reaction *in vitro*, to gain a further understanding of their metabolism during events leading to the acrosome reaction.

Materials and Methods

Media. The basic sperm capacitating medium (MCM-PL) was a Ca^{2+} -free minimal culture medium (Barros 1974) containing 106 mM-NaCl, 1.0 mM- MgCl_2 , 25.1 mM- NaHCO_3 , 0.25 mM-sodium pyruvate, 20 mM-sodium lactate, penicillin (100 units/ml) and streptomycin sulphate (50 $\mu\text{g/ml}$) prepared as described previously (Hyne & Garbers, 1979b). In some experiments 25 mM-Tris(hydroxymethyl)methyl-amine (Tris) replaced the NaHCO_3 in Medium MCM-PL and the pH was adjusted to pH 8.3 (Tris-PL). In other experiments using the Tris-buffered medium (pH 8.3), the substrates pyruvate and lactate were omitted and replaced by 2 mM-glucose or 2 mM-pyruvate. Tyrode's medium (with no albumin) containing pyruvate, lactate and glucose as substrates was prepared as described by Mujica & Valdes-Ruiz (1983).

The media were stored at 4°C and used in experiments within 1 week after preparation.

Sperm preparation and incubation procedure. Adult male guinea-pigs (Dunkin-Hartley or Monash University outbred strain) of > 700 g weight were anaesthetized with ether and killed by cervical dislocation. The spermatozoa were obtained by back-flushing isolated and lanced epididymal tails with a solution, containing 150 mM-NaCl and 1 mM- MgCl_2 , injected with a syringe into the lumen of each vas deferens as described previously (Hyne & Garbers, 1981). The spermatozoa were washed once by centrifugation (700 g, 10 min) and resuspended in 150 mM-NaCl-1 mM- MgCl_2 solution. In the general incubation procedure to examine the ability of various

substrates to inhibit or support the sperm acrosome reaction, samples (0.1 ml) of the sperm suspension were added to 0.9 ml Medium MCM-PL in sterile polystyrene tubes with caps (Falcon Plastics No. 2003, Oxnard, CA, U.S.A.) to which aliquants of various sugars or glycolytic substrates had previously been added from concentrated stock solutions. In the experiments with monensin (0.1 ml) samples of the sperm suspension were added to 0.9 ml Medium MCM-PL (previously equilibrated with 5% CO₂), in polystyrene tubes with caps, to which monensin, ethanol (0.005 ml) or Ca²⁺ had previously been added, and were incubated at 25°C. All incubations were initiated by the addition of spermatozoa (5 × 10⁶ spermatozoa/ml, final concentration) to the assay mixtures. The tubes were placed horizontally on a shaker, rotating at 60 strokes per min, in an incubator at 37°C with air as the atmosphere (unless otherwise stated).

Determination of sperm motility and acrosome reaction. At various times during an incubation after the addition of Ca²⁺, aliquants of the sperm suspension were transferred to a ceramic ring slide (Clay Adams) and mounted under a coverslip. At least 100 spermatozoa were examined to estimate the percentage of motile cells within the entire population of spermatozoa and the percentage of cells without a visible acrosomal cap within the motile sperm population by phase-contrast microscopy at × 160.

ATP assay. ATP was extracted from the spermatozoa by heat inactivation using a procedure described by Suter, Chow & Martin (1979). Aliquants (0.8 ml) of the final sperm suspensions were added to stoppered test tubes containing 2.2 ml of pre-heated distilled water in a heating block set at 95°C. The samples were heated for 10 min, then were transferred to an ice bath and subsequently centrifuged at 2000 g for 10 min at 4°C. The supernatant fluids were then stored at -80°C until assayed.

ATP was assayed by luminescence with purified luciferin-luciferase using a Packard Tri Carb Liquid scintillation spectrometer (model 3320) to measure the luminescence generated at 24°C (Stanley & Williams, 1969; Hammerstedt, 1973). The supplied luciferin-luciferase was reconstituted in a solution containing 1% bovine serum albumin and 10% glycerol (3 mg/ml) and dispensed from a light-shielded bottle. The sperm extract (0.5 ml), water (0.5 ml) and a buffer solution containing 40 mM-glycylglycine (pH 7.4) and 3 mM-MgSO₄ (1.0 ml) were added to clean and previously unused glass scintillation vials. Standard concentrations of ATP (0–160 pmol) were prepared in distilled water and prepared for assay using the same procedure as used for the sperm extracts. The reaction was initiated by the addition of 50 µl of the luciferin-luciferase solution and luminescence was recorded using out-of-coincidence counting over a 5 sec period (Stanley & Williams, 1969).

Chemicals. Luciferin-luciferase (L-0633), 2-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose (D-glucosamine), 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine), 2-amino-2-deoxy-D-galactose (D-galactosamine), 3-O-methyl-D-glucopyranose (3-O-methyl-glucose), DL-α-glycerophosphate, *myo*-inositol, sorbitol and D-gluconic acid were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Stock 10 mM or 100 mM solutions of these substrates were prepared in distilled water and were stored at -20°C until use, except 2-amino-2-deoxy-D-glucose which was prepared fresh before each experiment. A stock solution of monensin (10 mM) in ethanol was stored at -20°C. All other common chemicals were purchased from BDH (Australia) or Ajax (Australia).

Results

Effect of various sugars on the sperm acrosome reaction

Guinea-pig spermatozoa incubated in Medium MCM-PL capacitate in the absence of Ca²⁺ as indicated by their ability to respond to added Ca²⁺ with acrosome reactions within 10 min after a preincubation period of > 30 min (Hyne & Garbers, 1979b, 1981, 1982). After a 2–3 h incubation in

this medium, approximately 55–70% of the motile spermatozoa respond to added Ca^{2+} with acrosome reactions.

The effect of the presence of various sugars or glycolytic metabolites on the acrosome reaction when spermatozoa were incubated in Medium MCM-PL was investigated (Table 1). After incubation for 2–3 h, D-glucose (2 mM) slightly inhibited the guinea-pig sperm acrosome reaction in response to the addition of Ca^{2+} as reported previously (Rogers & Yanagimachi, 1975; Rogers, Chang & Yanagimachi, 1979). However, the 2-deoxy-analogues of D-glucose, 2-deoxy-D-glucose (2 mM) and 2-amino-2-deoxy-D-glucose (2 mM), completely inhibited acrosome reactions of motile spermatozoa, but the *N*-acetyl derivative 2-acetamido-2-deoxy-D-glucose (2 mM) had no inhibitory effect (Table 1). The substrates DL- α -glycerophosphate (2 mM) and sorbitol (2 mM) which have been reported to be metabolized by guinea-pig spermatozoa (Frenkel, Peterson & Freund, 1975) did not inhibit the sperm acrosome reaction and DL- α -glycerophosphate (2 mM) did not overcome the inhibitory effect of 2-deoxy-D-glucose. Similarly, the addition of *myo*-inositol (2 mM) or D-gluconic acid (2 mM) did not inhibit the acrosome reactions of motile spermatozoa incubated in Medium MCM-PL (Table 1). All treatments showed a similar percentage of motile spermatozoa over the 2–3 h incubation period.

Table 1. The effects of various sugars on the acrosome reaction of guinea-pig spermatozoa incubated in Medium MCM-PL

Treatment	% Acrosome reaction
Control	64 $\pm$ 3
D-Glucose	46 $\pm$ 17
2-Deoxyglucose	< 1
2-Glucosamine	< 1
<i>N</i> -Acetyl-2-glucosamine	70 $\pm$ 16
2-Amino-2-deoxy-D-galactose	31 $\pm$ 15
3-O-Methyl-D-glucose	74 $\pm$ 3
DL- α -Glycerophosphate	56 $\pm$ 5
DL- α -Glycerophosphate + 2-deoxyglucose	< 1
<i>Myo</i> -inositol	62 $\pm$ 3
<i>Myo</i> -inositol + 2-deoxyglucose	< 1
Sorbitol	59 $\pm$ 4
D-Gluconic acid	67 $\pm$ 2

Spermatozoa were incubated in Medium MCM-PL in the presence of the various substrates indicated (2 mM) for 2–3 h. Ca^{2+} (5 mM, final conc.) was added to the sperm suspensions for the final 10 min of the incubation. The percentage of motile spermatozoa without acrosomal caps was then assessed. The percentages of motile spermatozoa were not different for any of the treatments and varied from 50 to 65%. Values shown are the mean $\pm$ s.e.m. for 3 animals.

Factors influencing the inhibitory action of 2-deoxy-D-glucose

The effect of various concentrations of 2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose (glucosamine) on the Ca^{2+} -induced acrosome reaction was assessed on spermatozoa incubated for 3 h in Medium MCM-PL (Text-fig. 1). 2-Deoxy-D-glucose was a more effective inhibitor of the percentage of motile spermatozoa undergoing acrosome reactions than glucosamine, with concentrations of 2-deoxy-D-glucose $\geq 100 \mu\text{M}$ completely inhibiting the occurrence of the sperm acrosome reaction in response to the addition of 5 mM- Ca^{2+} . The inhibitory action of 2-deoxy-D-glucose (100 μM) on the acrosome reaction assessed at 3 h was only completely effective if the 2-deoxy-D-glucose was added to the spermatozoa suspended in Medium MCM-PL within 30 min of the start of the incubation. Spermatozoa treated with 2-deoxy-D-glucose (100 μM) after 0.5, 1, 1.5, 2

or 2.5 h and then incubated for a total of 3 h gave 2 ± 2 , 17 ± 8 , 29 ± 17 , 28 ± 12 and $56 \pm 6\%$ ($n = 4$) acrosome reactions. Thus, 2-deoxy-D-glucose (100 μM) added after 30 min did not prevent the motile spermatozoa from undergoing acrosome reactions at 3 h and did not inhibit the percentage of motile spermatozoa (50–60% after 3 h in all treatments), but the acrosome-reacted spermatozoa under these conditions did not show the vigorous motility change normally associated with an acrosome reaction (Yanagimachi & Usui, 1974). When guinea-pig spermatozoa incubated in Medium MCM-PL containing 2-deoxy-D-glucose (100 μM) for 135 min were washed twice by centrifugation and resuspended in Medium MCM-PL before the addition of 5 mM- Ca^{2+} , the percentage of acrosome reactions was $< 10\%$ compared with a control not treated with 2-deoxy-D-glucose which gave 75% acrosome reactions. In contrast, when spermatozoa were treated with low concentrations of 2-deoxy-D-glucose (100 or 200 μM) for an extended incubation period, before the addition of Ca^{2+} (5 mM), the inhibitory effect of 2-deoxy-D-glucose was overcome (Text-fig. 2). The addition of 8-Br-ATP (5 mM) or 8-Br-cAMP (5 mM) to spermatozoa suspended in Medium MCM-PL containing 2-deoxy-D-glucose (150 μM) did not overcome the inhibitory effect of 2-deoxy-D-glucose in preventing the acrosome reaction (data not shown). The addition of Na_2HPO_4 (1 mM) alone or in treatments containing the 8-bromo-adenosine nucleotides did not prevent 2-deoxy-D-glucose from inhibiting the sperm acrosome reaction.

Text-fig. 1. The effect of different concentrations of 2-deoxy-D-glucose or 2-glucosamine on the guinea-pig sperm acrosome reaction. Spermatozoa suspended in Medium MCM-PL were incubated with the inhibitor for 3 h; Ca^{2+} (5 mM, final conc.) was added for the final 10 min of the incubation and the percentage of acrosome reactions was then assessed. The percentage of motile spermatozoa was not different for any of the treatments and varied from 50 to 60%. Values are the mean $\pm$ s.e.m. for 3 observations.

Sperm ATP content and acrosome reactions in the presence of various substrates

Spermatozoa were incubated in Tris-buffered media (pH 8.3) containing glucose (2 mM) or pyruvate (2 mM) or both pyruvate (0.25 mM) and lactate (20 mM) as substrates and Medium MCM-PL, with 2-deoxy-D-glucose (2 mM) included in some treatments. The ATP content of the spermatozoa was measured 2 min and 2 h after the start of incubation and the percentage of motile spermatozoa and percentage acrosome reaction were assessed after 2 h in response to the addition of Ca^{2+} . Acrosome reactions were only observed with spermatozoa incubated in media containing

Text-fig. 2. Effect of incubation time on the inhibitory action of 2-deoxy-D-glucose on the guinea-pig sperm acrosome reaction. Spermatozoa were incubated for the various times indicated in Medium MCM-PL alone (○, ●), Medium MCM-PL with 100 μM-2-deoxy-D-glucose (△, ▲) or Medium MCM-PL with 200 μM-2-deoxy-D-glucose (□, ■). Ca^{2+} (5 mM, final conc.) was added to the sperm suspensions 10 min before determination of the percentage of motile spermatozoa (●, ▲, ■) or percentage of acrosome reactions (○, △, □). Each value is the mean $\pm$ s.e.m. for 3 animals.

Table 2. The effects of various substrates on the ATP content and acrosome reaction of guinea-pig spermatozoa incubated in bicarbonate- and Tris-buffered media

Treatment	ATP (nmol/ 10^8 spermatozoa)		% Acrosome reaction	% Motility
	2 min	120 min		
MCM-PL	23.5 ± 1.8	22.7 ± 7.4	63 ± 10	57 ± 3
MCM-PL + 2 mM-2-deoxy-glucose	17.0 ± 5.3	2.4 ± 0.5	<1	63 ± 2
Tris-PL	19.0 ± 1.4	14.5 ± 3.4	38 ± 11	58 ± 1
Tris-PL + 2 mM-2-deoxy-glucose	13.0 ± 2.5	1.3 ± 0.1	<1	58 ± 4
Tris + 2 mM-pyruvate	24.7 ± 0.8	4.1 ± 1.0	<1	60 ± 6
Tris + 2 mM-glucose	19.1 ± 3.1	7.8 ± 2.6	<1	59 ± 3

Spermatozoa were incubated with the substrates indicated for 2 h and at 2 and 120 min aliquants of the sperm suspension were removed to determine the sperm ATP concentration. After 2 h, Ca^{2+} (5 mM, final conc.) was added and 10 min later the percentage motility and the percentage of motile spermatozoa without acrosomal caps were assessed. Values shown are the mean $\pm$ s.e.m. for 3 animals.

both pyruvate (0.25 mM) and lactate (20 mM) as substrates and with no added 2-deoxy-D-glucose (Table 2). These spermatozoa also had maintained high ATP concentrations after incubation for 2 h compared with spermatozoa incubated in media containing 2-deoxy-D-glucose (2 mM), glucose (2 mM) alone or pyruvate (2 mM) alone. The addition of 2-deoxy-D-glucose (2 mM) to spermatozoa incubated with pyruvate and lactate caused a significant decrease in the sperm ATP concentrations

after incubation for 2 h (2.4 ± 0.5 nmol/ 10^8 cells) and the decline in the ATP concentrations appeared to be rapid as they were reduced after incubation for 2 min (Table 2). Prolonged incubation of spermatozoa treated with 2-deoxy-D-glucose (100 μ M) in Medium MCM-PL produced a further reduction in the sperm ATP concentrations (0.9 ± 0.5 nmol/ 10^8 cells, $n = 4$, after 5 h and 0.7 ± 0.1 nmol ATP/ 10^8 cells, $n = 4$, after 6 h).

In the presence of D-glucose as the only substrate, guinea-pig spermatozoa only undergo acrosome reactions after an extended incubation period (Rogers & Yanagimachi, 1975; Hyne & Garbers, 1981). Acrosome reactions occurred in Tris-buffered medium with pyruvate and lactate as substrates, but were reduced compared with values in bicarbonate-buffered medium (Table 2).

Sperm ATP content during monensin-stimulated acrosome reactions

Spermatozoa collected in Ca^{2+} -free media composed of 150 mM-NaCl and 1 mM- MgCl_2 or 300 mM-sucrose and 1 mM- MgCl_2 were transferred to Medium MCM-PL which was previously equilibrated with 5% CO_2 . In the presence of Ca^{2+} (5 mM) and monensin (50 μ M) the motile spermatozoa showed rapid acrosome reactions (20–40%) within 5–10 min (Text-fig. 3) as described previously (Hyne *et al.*, 1984; Hyne, 1984). Under these conditions, the sperm ATP concentration declined rapidly during the first 5 min of incubation and then stabilized at approximately 10% of the initial value. In comparison, in a treatment containing 0.5% ethanol and Ca^{2+} (5 mM), the sperm ATP concentration also initially declined rapidly within 5 min of the start of incubation, but the sperm ATP content then stabilized at a concentration 2–3-fold above the monensin-treated spermatozoa. Spermatozoa treated with monensin in the absence of added Ca^{2+} did not show a rapid decline in their ATP concentration, but rather a more gradual decrease in ATP concentration over the incubation period (Text-fig. 3). The spermatozoa in all treatments maintained good motility (60–70%) over the incubation period, but acrosome reactions were only observed in the

Text-fig. 3. Guinea-pig sperm ATP concentrations in the presence of monensin and extracellular Ca^{2+} . Spermatozoa were suspended at 25°C in Medium MCM-PL (pH 7.5) with 0.5% ethanol and 5 mM- Ca^{2+} (■), Medium MCM-PL (pH 7.5) with 50 μ M-monensin (▲) or Medium MCM-PL (pH 7.5) with 50 μ M-monensin and 5 mM- Ca^{2+} (●). At the various times indicated aliquants of the sperm suspension were removed to determine the sperm ATP concentration. The spermatozoa in all treatments maintained 60–75% motility over the incubation period. Acrosome reactions (20–40%) were only observed in the treatment containing 50 μ M-monensin and 5 mM- Ca^{2+} after 5 min incubation time. Values are the mean $\pm$ s.e.m. of 3 animals.

presence of both Ca^{2+} and monensin. If the spermatozoa were resuspended in media buffered with $\text{HCO}_3^-/\text{CO}_2$ (pH 7.5) similar in composition to Medium MCM-PL except that the substrates pyruvate and lactate were omitted and 5 mM-glucose or no substrates were utilized, then the addition of monensin (50 μM) inhibited the sperm motility within 10 min in the presence of 5 mM- Ca^{2+} (data not shown).

Discussion

These studies confirm previous observations that the metabolic state of the spermatozoon can influence and regulate the process of capacitation and the acrosome reaction (Rogers & Yanagimachi, 1975; Hoppe, 1976; Rogers, Ueno & Yanagimachi, 1977; Fraser & Quinn, 1981). Culture conditions that maintain high ATP concentrations in guinea-pig spermatozoa during incubation *in vitro* also support a short capacitation period and a rapid onset of the acrosome reaction. Media containing both pyruvate and lactate as substrates maintained higher concentrations of ATP in guinea-pig spermatozoa and supported a more rapid onset of acrosome reactions than did media containing pyruvate alone or glucose (Rogers & Yanagimachi, 1975; Hyne & Garbers, 1981). The 2-deoxy analogues of glucose, 2-deoxy-D-glucose and 2-amino-D-glucose, are very potent inhibitors of the acrosome reaction of guinea-pig spermatozoa when incubated with pyruvate and lactate as substrates, whereas the *N*-acylated derivative 2-acetamido-2-deoxy-D-glucose was ineffective. Both 2-deoxy-D-glucose and 2-amino-D-glucose are phosphorylated by hexokinase, while the *N*-acylated derivative is not phosphorylated (Webb, 1966; Hiipakka & Hammerstedt, 1978), suggesting that the phosphorylation of these sugars, and the resultant accumulation of the stable 6-phosphate metabolites, is responsible for their irreversible inhibitory action. Possible reasons for the potent inhibitory effect of 2-deoxy-D-glucose might include inhibition of phosphoglucose isomerase or other glycolytic enzymes, as well as depletion of the ATP concentrations. The addition of exogenous DL- α -glycerophosphate, which is metabolized by guinea-pig spermatozoa (Frenkel *et al.*, 1975), did not overcome the inhibitory action of 2-deoxy-D-glucose, suggesting that the inhibition produced by 2-deoxy-D-glucose was perhaps not due to depletion of some of the endogenous glycolytic metabolites, but to the diversion of intracellular phosphate to the stable 2-deoxy-D-glucose-6-phosphate and depletion of the cellular ATP concentrations.

2-Deoxy-D-glucose was only completely effective in preventing acrosome reactions if the sugar was added to the sperm suspension within 30 min of the start of incubation. The addition of 2-deoxy-D-glucose also appeared to decrease the sperm ATP concentrations rapidly. These data suggest that there may be an essential requirement for an ATP-dependent reaction, or phosphorylation of an unknown receptor, to be completed during capacitation before spermatozoa undergo the acrosome reaction. Prolonged incubation of spermatozoa treated with 2-deoxy-D-glucose partly overcame the block to the acrosome reaction, despite the reduced ATP concentrations.

The mechanisms involved in the sperm capacitation process may have analogies with the maturation and ageing of mammalian erythrocytes, whereby energy depletion retards the maturation-associated loss of membrane transport functions during reticulocyte maturation (Weigensberg & Blostein, 1983). The present study demonstrates that culture conditions that support a short capacitation period and a rapid onset of acrosome reactions also maintain elevated guinea-pig sperm ATP concentrations. These culture conditions also support a rapid Ca^{2+} and HCO_3^- -dependent rise in cyclic AMP concentrations (Hyne & Garbers, 1979b; Garbers, Tubb & Hyne, 1982) and lead to elevated sperm intracellular Na^+ concentrations (Hyne *et al.*, 1984). The induction of rapid acrosome reactions by the Na^+ -ionophore monensin is also dependent on extracellular Na^+ and Ca^{2+} at an optimal HCO_3^- concentration (Hyne, 1984) and in addition require the oxidative substrates pyruvate and lactate that maintain high guinea-pig sperm ATP concentrations.

The Ca^{2+} -dependent depletion of guinea-pig sperm ATP concentrations observed in this study may explain, in part, why an initial preincubation of guinea-pig spermatozoa in Ca^{2+} -free media supports a shorter capacitation period and more rapid onset of acrosome reactions (Hyne & Garbers, 1979b).

These observations contrast with those obtained by Santos-Sacchi & Gordon (1982) who induced synchronous acrosome reactions in guinea-pig spermatozoa by initially preincubating the spermatozoa in substrate-free medium to deplete the sperm ATP concentrations and then stimulating acrosome reactions by the addition of pyruvate. Glucose did not substitute for pyruvate in the procedure and the authors showed that the addition of pyruvate was essential to enable the ATP-depleted spermatozoa to resynthesize ATP. Maximal acrosome reactions occurred after 60 min of the addition of the pyruvate, suggesting that the ATP was necessary for the membrane fusion reaction. Santos-Sacchi & Gordon (1982) suggested that the initial preincubation of the spermatozoa in the substrate-free medium to deplete ATP concentrations led to a rapid capacitation due to inactivation of a Ca^{2+} -dependent ATPase expulsion mechanism for Ca^{2+} , thereby permitting Ca^{2+} influx. An alternative explanation is that the ATP-depletion of the spermatozoa would lead to elevated intracellular Na^+ concentrations, a rate-determining event previously associated with treatments that stimulate synchronous guinea-pig sperm acrosome reactions (Hyne *et al.*, 1984).

A novel procedure for inducing guinea-pig sperm acrosome reactions has been described by Mujica & Valdes-Ruiz (1983): the spermatozoa were preincubated in Tyrode's medium containing glucose as substrate and then transferred to a minimal culture medium containing pyruvate and lactate. The authors concluded that guinea-pig sperm capacitation occurred within a short time after incubation in Tyrode's medium and that capacitation was not delayed by glucose, but that the acrosome reaction was arrested by a glucose metabolite. In addition, Mujica & Valdes-Ruiz (1983) found that the capacitation of the spermatozoa incubated in Tyrode's medium was not pH-dependent, in contrast to the results of Hyne & Garbers (1981). The occurrence of acrosome reactions in the transferred spermatozoa was determined quantitatively by fixing the sperm suspension with formaldehyde and the results compared with spermatozoa incubated continuously in minimal culture medium containing pyruvate and lactate, but which were not carried through the centrifugation step (Mujica & Valdes-Ruiz, 1983). However, when guinea-pig spermatozoa were treated by this procedure and the occurrence of acrosome reactions quantitatively assessed by observation of wet mount aliquants of motile spermatozoa, we did not observe a significant percentage of acrosome reactions in the motile sperm population. The reason for this discrepancy is not readily apparent, but one possible cause may be the centrifugation procedure inducing a Ca^{2+} -dependent acrosome-loss in live but immotile spermatozoa, compared with the control treatments that were not centrifuged.

The reason high ATP concentrations are required for rapid sperm capacitation is unknown, but ATP or cyclic AMP-dependent phosphorylation of membrane lipid or protein in the acrosomal region at the site of membrane fusion may be involved. Attempts to identify phosphorylated sites in spermatozoa (Brandt & Hoskins, 1980; Bearer & Friend, 1982) have had only limited success because intact spermatozoa do not equilibrate extracellular and intracellular Pi (Babcock, First & Lardy, 1975). This explains why added phosphate did not overcome the inhibitory effect of 2-deoxy-D-glucose. It has been suggested that polyphosphoinositide may be responsible for Ca^{2+} sequestration in the guinea-pig acrosome (Friend, 1982; Bearer & Friend, 1982) and membrane fusion of myoblasts (Wakelam, 1983). Cyclic AMP-dependent protein kinase has been reported to stimulate indirectly the formation of polyphosphoinositides in the plasma membranes of different blood cells (Enyedi, Farago, Sarkadi, Szasz & Gardos, 1983). Alternatively, membrane phosphorylation may regulate the kinetic properties of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Reeves & Sutko, 1983; Rufo, Schoff & Lardy, 1984), to reverse the carrier system and cause Ca^{2+} influx which in turn stimulates the sperm acrosome reaction, when the sperm intracellular Na^+ values have risen to a critical value (Hyne, 1984). The data suggest that when guinea-pig spermatozoa are capacitated

by incubation in Medium MCM-PL, the ATP-dependent biochemical event is essentially complete within 1 h of incubation and sperm capacitation is then not inhibited by the addition of 2-deoxy-D-glucose. Direct evidence for the phosphorylation of an endogenous receptor during the acrosome reaction must await the development of a procedure to effectively label the sperm intracellular phosphate pools with a radiochemical marker.

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References

- Babcock, D.F., First, N.L. & Lardy, H.A. (1975) Transport mechanism for succinate and phosphate localized in the plasma membrane of bovine spermatozoa. *J. biol. Chem.* **250**, 6488–6495.
- Barros, C. (1974) Capacitation of mammalian spermatozoa. In *Physiology and Genetics of Reproduction*, Part B, pp. 3–24. Eds E. M. Coutinho & F. Fuchs. Plenum Press, New York.
- Bavister, B.D. (1981) Substitution of a synthetic polymer for protein in a mammalian gamete culture system. *J. exp. Zool.* **217**, 45–51.
- Bearer, E.L. & Friend, D.S. (1982) Modifications of anionic-lipid domains preceding membrane fusion in guinea pig sperm. *J. Cell Biol.* **92**, 604–615.
- Bedford, J.M. (1983) Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* **28**, 108–120.
- Brandt, H. & Hoskins, D.D. (1980) An AMP-dependent phosphorylated motility protein in bovine epididymal sperm. *J. biol. Chem.* **255**, 982–987.
- Davis, B.K. (1976) Influence of serum albumin on the fertilizing ability in vitro of rat spermatozoa. *Proc. Soc. exp. Biol. Med.* **151**, 240–243.
- Dravland, E. & Meizel, S. (1981) Stimulation of hamster sperm capacitation and acrosome reaction in vitro by glucose and lactate and inhibition by the glycolytic inhibitor α -chlorohydrin. *Gamete Res.* **4**, 515–523.
- Enyedi, A., Farago, A., Sarkadi, B., Szasz, I. & Gardos, G. (1983) Cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in the plasma membranes of different blood cells. *FEBS Letters* **161**, 158–162.
- Fraser, L.R. & Quinn, P.J. (1981) A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J. Reprod. Fert.* **61**, 25–35.
- Frenkel, G., Peterson, R.N. & Freund, M. (1975) Oxidative and glycolytic metabolism of semen components by washed guinea pig spermatozoa. *Fert. Steril.* **26**, 144–147.
- Friend, D.S. (1982) Neomycin/peroxidase/gold labeling of the guinea pig sperm acrosomal membrane. *J. Cell Biol.* **95**, 149, Abstr.
- Garbers, D.L., Tubb, D.J. & Hyne, R.V. (1982) A requirement of bicarbonate for Ca^{2+} -induced elevations of cyclic AMP in guinea pig spermatozoa. *J. biol. Chem.* **257**, 8980–8984.
- Hammerstedt, R.H. (1973) An automated method for ATP analysis utilizing the luciferin-luciferase reaction. *Analyt. Biochem.* **52**, 449–455.
- Hiipakka, R.A. & Hammerstedt, R.H. (1978) 2-Deoxy-glucose transport and phosphorylation by bovine sperm. *Biol. Reprod.* **19**, 368–379.
- Hoppe, P.C. (1976) Glucose requirement for mouse sperm capacitation in vitro. *Biol. Reprod.* **15**, 39–45.
- Hoppe, P.C. & Whitten, W.K. (1974) An albumin requirement for fertilization of mouse eggs in vitro. *J. Reprod. Fert.* **39**, 433–436.
- Hoshi, K., Saito, A., Suzuki, M., Hayashi, K. & Yanagimachi, R. (1982) Effects of substrates on penetration of human spermatozoa into the zona pellucida of human eggs and the zona-free hamster eggs. *Jap. J. Fert. Steril.* **27**, 439–444.
- Hyne, R.V. (1984) A bicarbonate and calcium dependent induction of rapid guinea-pig sperm acrosome reactions by monovalent ionophores. *Biol. Reprod.* **31**, 312–323.
- Hyne, R.V. & Garbers, D.L. (1979a) Regulation of guinea pig sperm adenylate cyclase by calcium. *Biol. Reprod.* **21**, 1135–1142.
- Hyne, R.V. & Garbers, D.L. (1979b) Calcium dependent increase in adenosine 3',5'-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5699–5703.
- Hyne, R.V. & Garbers, D.L. (1981) Requirement of serum factors for capacitation and the acrosome reaction of guinea pig spermatozoa in buffered medium below pH 7.8. *Biol. Reprod.* **24**, 257–266.
- Hyne, R.V. & Garbers, D.L. (1982) Inhibition of the guinea-pig sperm acrosome fraction by a low molecular weight factor(s) in epididymal fluid and serum. *J. Reprod. Fert.* **64**, 151–157.
- Hyne, R.V., Higginson, R.E., Kohlman, D. & Lopata, A. (1984) Sodium requirement for capacitation and membrane fusion during the guinea-pig sperm acrosome reaction. *J. Reprod. Fert.* **70**, 83–94.
- Lopata, A., McMaster, R., McBain, J.C. & Johnston, W.I.H. (1978) In-vitro fertilization of preovulatory human eggs. *J. Reprod. Fert.* **52**, 339–342.
- Lui, C.W., Cornett, L.E. & Meizel, S. (1977) Identification of the bovine follicular fluid protein involved in the in vitro induction of the hamster sperm acrosome reaction. *Biol. Reprod.* **17**, 34–41.
- Mahi, C.A. & Yanagimachi, R. (1976) Maturation and sperm penetration of canine ovarian oocytes in vitro. *J. exp. Zool.* **196**, 189–195.
- Mahi, C.A. & Yanagimachi, R. (1978) Capacitation, acrosome reaction and egg penetration by canine spermatozoa in a simple defined medium. *Gamete Res.* **1**, 101–109.
- Mrsny, R.J., Waxman, L. & Meizel, S. (1979) Taurine

- maintains and stimulates motility of hamster sperm during capacitation *in vitro*. *J. exp. Zool.* **210**, 123–128.
- Mujica, A. & Valdes-Ruiz, M.A. (1983) On the role of glucose in capacitation and acrosomal reaction of guinea pig sperm. *Gamete Res.* **8**, 335–344.
- Niwa, K. & Chang, M.C. (1975) Requirement of capacitation for sperm penetration of zona-free rat eggs. *J. Reprod. Fert.* **44**, 305–308.
- Oliphant, G. (1976) Removal of sperm bound seminal plasma components as a prerequisite to induction of the rabbit acrosome reaction. *Fert. Steril.* **27**, 28–38.
- Oliphant, G. & Singhas, C.A. (1979) Iodination of rabbit sperm plasma membrane: relationship of specific surface proteins to epididymal function and sperm capacitation. *Biol. Reprod.* **21**, 937–944.
- O'Rand, M.G. (1979) Changes in sperm surface properties correlated with capacitation. In *The Spermatozoon: Maturation, Motility, Surface Properties and Comparative Aspects*, pp. 195–204. Eds D. W. Fawcett & J. M. Bedford. Urban and Schwarzenberg, Baltimore.
- Perreault, S.D. & Rogers, B.J. (1981) Effect of various sugars on the time course of human spermatozoal capacitation *in vitro*. *J. Androl.* **2**, 22, Abstr.
- Quinn, P., Stanger, J.D. & Whittingham, D.G. (1982) Effect of albumin on fertilization of mouse ova *in vitro*. *Gamete Res.* **6**, 305–313.
- Reeves, J.P. & Sutko, J.L. (1983) Competitive interactions of sodium and calcium with the sodium – calcium exchange system of cardiac sarcolemmal vesicles. *J. biol. Chem.* **258**, 3178–3182.
- Rogers, B.J. (1978) Mammalian sperm capacitation and fertilization *in vitro*: a critique of methodology. *Gamete Res.* **1**, 165–223.
- Rogers, B.J. & Yanagimachi, R. (1975) Retardation of guinea pig sperm acrosome reaction by glucose: the possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction. *Biol. Reprod.* **13**, 568–575.
- Rogers, B.J., Ueno, M. & Yanagimachi, R. (1977) Inhibition of hamster sperm acrosome reaction and fertilization by oligomycin, antimycin A and rotenone. *J. exp. Zool.* **199**, 129–135.
- Rogers, B.J., Chang, L. & Yanagimachi, R. (1979) Glucose effect on respiration: possible mechanism for capacitation in guinea pig spermatozoa. *J. exp. Zool.* **207**, 107–112.
- Rufo, G.A., Jr, Schoff, P.K. & Lardy, H.A. (1984) Regulation of calcium content in bovine spermatozoa. *J. biol. Chem.* **259**, 2547–2552.
- Saling, P.M. & Bedford, J.M. (1981) Absence of species specificity for mammalian sperm capacitation *in vivo*. *J. Reprod. Fert.* **63**, 119–123.
- Santos-Sacchi, J. & Gordon, M. (1982) The effect of ATP depletion upon the acrosome reaction in guinea-pig sperm. *J. Androl.* **3**, 108–112.
- Stanley, P.E. & Williams, S.G. (1969) Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Analyt. Biochem.* **29**, 381–392.
- Suter, D., Chow, P.Y.W. & Martin, I.C.A. (1979) Maintenance of motility in human spermatozoa by energy derived through oxidative phosphorylation and addition of albumin. *Biol. Reprod.* **20**, 505–510.
- Tsunoda, Y. & Chang, M.C. (1975) In-vitro fertilization of rat and mouse eggs by ejaculated sperm and the effect of energy sources on in-vitro fertilization of rat eggs. *J. exp. Zool.* **193**, 79–86.
- Wakelam, M.J.O. (1983) Inositol phospholipid metabolism and myoblast fusion. *Biochem. J.* **214**, 77–82.
- Webb, J.L. (1966) Analogs of enzyme reaction components: hexokinases. In *Enzyme and Metabolic Inhibitors*, Vol. 2, pp. 376–405. Academic Press, New York.
- Weigensberg, A.M. & Blostein, R. (1983) Energy depletion retards the loss of membrane transport during reticulocyte maturation. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4978–4982.
- Yanagimachi, R. (1972) Fertilization of guinea pig eggs *in vitro*. *Anat. Rec.* **174**, 9–20.
- Yanagimachi, R. & Usui, N. (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Expl Cell Res.* **89**, 161–174.
- Yanagimachi, R., Yanagimachi, H. & Rogers, B.J. (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol. Reprod.* **15**, 471–476.

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