Introduction
Spermatozoa require ATP for many purposes but most ATP is directed towards motility (Bohnsack and Halangk, 1986; Ford and Rees, 1990; Mita et al., 1991; Hofmann et al., 1992). Although the metabolic substrates used for ATP generation by spermatozoa vary among species, mature boar spermatozoa are dependent upon certain glycolysable substrates, lactate and, to a limited extent, short-chain fatty acids such as acetate and propionate (Jones and Chantrill, 1989). Thus boar spermatozoa are almost totally reliant on the glycolytic pathway, not for the direct production of ATP by substrate-level phosphorylation, but for the production of lactate which is the primary source of mitochondrial acetyl CoA (Jones, 1997).

Spermatozoa from rams (Tash and Mann, 1973) and rhesus monkeys (Hoskins and Patterson, 1968) maintain their motility when incubated aerobically in substrate-free media. Furthermore, Jones and Milmlow (1997) confirmed earlier observations (Kamp et al., 1996) that washed boar spermatozoa are also capable of maintaining their metabolic integrity when incubated for up to 5 h in the absence of any exogenous substrates. Two endogenous substrates were identified, which are mobilized to produce the triosephosphates that allow lactate synthesis to be maintained. One substrate produced glycerol 3-phosphate, which was converted to dihydroxyacetone phosphate by the FAD-linked mitochondrial glycerol 3-phosphate dehydrogenase (Jones and Gillan, 1996a,b) and the other degraded to glycerol, which indicated that it may be a di- or tri-glyceride. Acetylcarnitine contributes acetyl groups early in the incubation; glycerylphosphorylcholine is degraded to glycerol 3-phosphate and choline after about 5 h, and acetate also accumulates after about 5 h. The presence of phosphorylcholine and phosphorylethanolamine later in the incubation indicates that phospholipids are also degraded to glycerol.

Materials and Methods
Chemicals and substrates
3-Bromopyruvate hydrate (Aldrich Chemical Co., Milwaukee, WI) was recrystallized several times from dry chloroform (melting point 54–57°C). Aqueous solutions were neutralized with solid NaHCO3 and added to incubations as sodium bromopyruvate (BPA) at a final concentration of 0.5 mmol l−1. (R,S)-a-bromohydrin phosphate biscyclohexylamine salt was prepared to established purity by the method of Jones and Gillan (1996a). All enzymes and substrates were supplied by Boehringer Mannheim (Castle Hill, NSW) and compounds were prepared as aqueous solutions and used at concentrations indicated in the text. All other chemicals and reagents were of analytical grade and all solutions were prepared in water purified by reverse osmosis.

Collection and preparation of spermatozoa
Cauda epididymides were removed from the testis-epididymis complex of mature boars (Sus scrofa: various crosses of Duroc, Hampshire, Landrace and Large White) 15–20 min after the animals were killed at an abattoir. The cauda epididymides were placed immediately in Dewar flasks containing PBS at 34°C. PBS consisted of NaCl (139 mmol l−1), KCl (4.7 mmol l−1), MgSO4 (1.2 mmol l−1), Na2HPO4 (3.1 mmol l−1), NaH2PO4 (0.8 mmol l−1) and CaCl2 (2.5 mmol l−1), adjusted to pH 7.4. The sealed flasks were transported to metabolic inhibitors. This experiment was complemented by NMR investigations, which aided the identification of several substrates used as metabolic fuels by the cells under these conditions.

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the laboratory where the spermatozoa were flushed with PBS at 34°C from incisions made in the third and fourth segments of the cauda epididymidis according to the classification of Holtz and Smidt (1976). The sperm suspension, usually from 10–20 boars, was centrifuged (2000 g for 10 min), the supernatant solution discarded and the cells aspirated from any denser erythrocytes and resuspended in PBS at 34°C. This procedure was repeated twice and the spermatozoa were finally prepared as a 10% (v/v) suspension in PBS at 34°C. The concentration of spermatozoa in this suspension was approximately 10^7 cells ml^-1 containing 15–25 mg protein ml^-1.

**Incubations with spermatozoa**

Incubations were performed at 34°C in either 25 ml (short-term) or 50 ml (long-term) open conical flasks shaken at 120 cycles min^-1 with air as the gas phase. Long-term incubations contained 35 ml (approximately 3–4 × 10^6 cells) of the 10% sperm suspensions with no exogenous substrates and, where applicable, 1 ml of the appropriate inhibitor. Samples (1 ml) were removed at time 0 and at intervals of 1 h and added to HClO₄ (3 mol l⁻¹, 100 μl). Short-term incubations were commenced at time 0 and at intervals of 1 h by taking 5 ml (approximately 5 × 10^6 cells) of the long-term incubations, adding this to the appropriate inhibitor (250 μl) to a final concentration as specified in the text and subsequently maintaining the samples for 1 h. Samples (1 ml) of the short-term incubations were removed every 15 min for the next hour and added to HClO₄ (3 mol l⁻¹, 100 μl). Deproteinized, neutralized and desalted incubation solutions (Stevenson and Jones, 1982) were used for assays of glycerol 3-phosphate and lactate (Lang, 1985), ATP (Jaworek and Welsch, 1985a), ADP and AMP (Jaworek and Welsch, 1985b) and glycerol (Wieland, 1984), using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto). Protein was estimated (Lowry et al., 1951) on freeze–thawed samples using BSA as the standard. The adenine nucleotide pool is defined as [ATP] + [ADP] + [AMP] and energy charge potentials (ECPs) were calculated using the method of Atkinson and Walton (1967), according to the following equation:

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ECP = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}
\]

**Saponification of sperm pellets**

Sperm suspensions were incubated for 10 h in the absence of exogenous substrates and samples (1 ml) were removed every hour and added to pre-weighed centrifuge tubes. After centrifugation (2000 g for 10 min) and removal of the supernatant solution, the wet pellets were weighed and saponified at 70°C for 15 min with KOH (1 mol l⁻¹, 3 ml). Ethanol (95%, 2 ml) was added and heating was continued for a further 2 h. H₂SO₄ (9 mol l⁻¹, 0.5 ml) was added to the cooled mixture and, after centrifugation (2000 g for 5 min), the decanted supernatant solutions were neutralized, desalted and assayed for glycerol.

**Estimation of triglycerides**

Sperm suspensions were incubated for 10 h in the absence of exogenous substrates and samples (1 ml) were removed every hour, added to HClO₄ (3 mol l⁻¹, 100 μl) and frozen until assayed. Triglycerides were extracted from the thawed samples by a modification of the procedure of Bryson et al. (1995). A sample of the acidified suspension (500 μl) was added to 10 ml chloroform:methanol (2:1, v/v) in a Potter-Elvehjem homogenizer and the mixture was kept at room temperature for 3 h with occasional agitation. The mixture was transferred to a 15 ml screw-capped tube with 0.9% NaCl (2 ml), shaken and centrifuged (2000 g for 0.5 min). The lower chloroform phase was transferred to a clean tube and allowed to evaporate. The residue was dissolved in ethanol (500 μl) and an aliquot (50 μl) was assayed using the triglycerides GPO-PAP assay kit (Boehringer Mannheim).

**Estimation of fatty acids**

Sperm suspensions were incubated for 5 h in the absence of exogenous substrates and samples (1 ml) were removed at 0 and 5 h and added to HClO₄ (3 mol l⁻¹, 100 μl). The samples were deproteinized, neutralized and desalted, and aliquots (50 μl) were methylated using the method of Lepage and Roy (1986). Fatty acid methyl esters were assayed on a Hewlett Packard 5890A capillary gas chromatograph with flame ionization detection with a 30 m × 0.25 mm i.d. DB-225 on fused carbon silica column (J and W Scientific, Folsom, CA) and a two-step heating programme (170°C for 2 min, 10°C min⁻¹ to 190°C; 190°C for 1 min, 5°C min⁻¹ to 220°C). Retention times were compared with those of standard fatty acid methyl esters in GLC 68 (Nuchek prep, Elysian, MN).

**NMR studies**

Sperm suspensions were incubated for 10 h in the absence of exogenous substrates and samples (2 ml) were removed every hour, added to HClO₄ (3 mol l⁻¹, 200 μl), deproteinized, neutralized, desalted and adjusted to 3 ml. NMR assignments were made from spectra acquired for solutions obtained by lyophilization of these 3 ml samples and redissolving the residue in D₂O (550 μl). Changes in metabolite concentrations were monitored by acquiring 1H spectra for unprocessed residue in D₂O (550 μl). Changes in metabolite concentrations were monitored by acquiring 1H spectra for unprocessed 1H spectra for unprocessed solutions (550 μl) dispensed in NMR tubes, with a coaxial capillary containing 1 mmol l⁻¹ sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d₄, sodium salt; Commissariat à l’Énergie Atomique, Gif-sur-Yvette, Cedex) as chemical shift and intensity reference in D₂O as field-frequency lock.

NMR spectra were obtained with a Bruker AMX-600 spectrometer, using a broadband inverse-detection xyz-gradient probe at a 1H frequency of 600.14 MHz and the variable temperature unit set to 25°C. Assignments were made with data from proton–proton (COSY; Derome and I’Énergie Atomique, Gif-sur-Yvette, Cedex) as chemical shift and intensity reference in D₂O as field-frequency lock.
saturation (2 s) as described by Urashima et al. (1994). Spectral widths were routinely for 1H, 5 kHz, and for 13C, 16.6 kHz in one-bond and 31 kHz in multiple-bond C–H correlation experiments (HMBC, Bax and Summers, 1986). Chemical shifts are expressed relative to the methyl resonance of choline (1H, δ 3.21; 13C, δ 55.0) (Nicholson et al., 1995) and represent the mid-points of cross-peaks in the HMBC experiments.

Comparisons of metabolite concentrations were obtained from fully relaxed 1H spectra acquired over 64K data points for a spectral width of 6010 Hz; solvent pre-saturation for 4 s followed a relaxation delay of 21 s. Compounds were identified by assignment of all detectable correlations in both homonuclear and heteronuclear experiments and comparison of the designated 1H and 13C chemical shifts with published data (Dijkstra et al., 1985; Malik et al., 1992; Nicholson et al., 1995; Moreno and Arús, 1996; Kalic et al., 1997; Bubb et al., 1999). 2D NMR studies were performed on an unincubated sample (0 h) and one that had been incubated for 10 h at 34°C to determine the overall pattern of changes.

**Statistical analyses**

Values are presented as the mean ± SEM for the number (n) of experiments performed. When appropriate, statistical significance was tested using Student’s unpaired t-test with a two-tailed distribution.

**Results**

Washed boar spermatozoa maintained a reasonably high ECP (0.5–0.8) over 10 h when incubated at 34°C in the absence of exogenous substrates (Fig. 1a). Addition at any time of 3-bromopyruvate (BPA), which inhibits stage 2 of the glycolytic pathway (Jones et al., 1995), caused an immediate decrease in the ECP. In incubations containing BPA at time 0, the ECP rapidly decreased over the first few hours, but showed a gradual increase from about 5 h (Fig. 1b). When incubated in the presence of (R,S)-α-bromohydrin phosphate, an inhibitor of the FAD-linked mitochondrial glycerol 3-phosphate dehydrogenase (Jones and Gillan, 1996a), the ECP decreased steadily over the first 5 h but showed some slight increase at 9 h (Fig. 1c). At any time during this incubation, addition of BPA caused an immediate decrease in ECP. This decrease was less marked at the start of the incubation; the ECP during the first hour was higher (P < 0.05) than during the second hour, indicating the early mobilization of a substrate or substrates that did not require processing by stage 2 of the glycolytic pathway.

Washed boar spermatozoa maintained a concentration of lactate usually of the order of 15–30 nmol mg−1 protein in the presence of a metabolizable substrate, but when spermatozoa were incubated at 34°C with no exogenous substrate, this value decreased to 5–8 nmol mg−1 protein within 2 h and was maintained at about 2–4 nmol mg−1 protein for the duration of the incubation (Fig. 2a). Saponification of sperm pellets from incubations performed in the presence of (R,S)-α-bromohydrin phosphate showed that the concentration of glycerol decreased rapidly over the first hour and remained at about 50% of values at time 0 for up to 10 h (Fig. 2b). Although glycerol 3-phosphate was not detected in suspensions incubated for 5 h (Jones and Milmlow, 1997), it was detected in the present study when the incubation was carried out in the presence of (R,S)-α-bromohydrin phosphate. Furthermore, under these conditions, the concentration of glycerol 3-phosphate increased beyond 4 h to almost three times the value at time 0 (Fig. 2c). At time 0, sperm suspensions assayed for triglycerides at 40–69 nmol mg−1 protein (n = 6), whereas after 5 h incubation in the absence of exogenous substrate, this decreased to 8–17 nmol mg−1 protein (n = 6). At time 0, the long-chain fatty acids present in one suspension comprised C16:0, C 18:0 and C 18:1 and by 5 h the amounts had almost doubled, comprising mainly C16:0, C18:0, C18:1 and C20:1.

The 2D NMR study identified a number of low molecular mass compounds present in the incubates (Table 1), although not all resonances (such as the CH of lactate) were observed for certain metabolites present in low concentrations. Those metabolites that were detected both at 0 h and 10 h included choline, carnitine, hypotaurine and betaine (N,N,N-trimethylglycine); those only detected at 0 h were lactate, glutamine, acetyl/carnitine and glycerol phosphate; and those that were only detected at 10 h were glucose, lysine, ethanolamine,
phosphorylcholine and phosphorylethanolamine. Only traces of acetate remained after freeze-drying but it was apparent from the $^1$H spectra obtained for unprocessed samples that the time course of acetate enrichment was similar to that for the appearance of choline. The loss of glycerylphosphorylcholine, and the subsequent appearance of choline, was followed over the 10 h period (Fig. 3).

### Discussion

Washed boar spermatozoa incubated in the presence of a number of substrates *in vitro* can only oxidize glucose, fructose, glycerol, glycerol 3-phosphate, lactate and short-chain fatty acids (Jones and Chantrill, 1989). Apart from the short-chain fatty acids, the carbon atoms of these substrates are channelled...
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Conversion of glycerylphosphorylcholine to choline in boar spermatozoa incubated without exogenous substrate at 34°C for 10 h. (a) Lactate (n = 4). (b) Saponifiable glycerol in the presence of (R,S)-α-bromohydrin phosphate (α-BrP, 5 mmol l⁻¹). Values are given as a percentage of those at time 0 (n = 6). (c) Glycerol 3-phosphate in the presence of α-BrP (5 mmol l⁻¹)(n = 6).

Fig. 3. Conversion of glycerylphosphorylcholine to choline in boar spermatozoa incubated without exogenous substrate at 34°C over 10 h. The 600 MHz ¹H NMR-N+(CH₃)₃ resonances of glycerylphosphorylcholine (GPC) and choline (CHO) over the 10 h incubation period. The resonance due to carnitine (CAR) is almost coincident with that of GPC. The concentration of GPC + CAR was approximately 2.5 mmol l⁻¹ from 0 to 4 h and 0.2 mmol l⁻¹ from 5 to 10 h; the concentration of CHO increased to 0.4 mmol l⁻¹ after 7 h.

towards the production of lactate, which is the metabolic fuel for the mitochondrial production of ATP (Jones, 1997). However, the substrates used by spermatozoa in vitro are unknown. At present, information is based predominantly on experiments with washed sperm suspensions replenished with sugars or other metabolizable substrates; however, ejaculated spermatozoa are not exposed to the artificial conditions created during examination in vitro (Mann and Lutwak-Mann, 1981). Although incubation of spermatozoa in the absence of any exogenous substrate is an artificial condition, it has been demonstrated that washed boar spermatozoa incubated for up to 5 h with no exogenous substrate maintain the potential to synthesize ATP (Jones and Milmlow, 1997). Specific metabolic inhibitors revealed that the cells produced fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glycerol, indicating that mobilization of unidentified endogenous compounds was occurring and that these were producing the triosephosphates. However, the question remained as to what compounds were being mobilized and what substrates were being produced.

Two endogenous components depleted early in the 10 h incubation are acetylcarnitine and di- and tri-glycerides. Acetylcarnitine and carnitine are present in boar epididymal spermatozoa (Casillas, 1972). It has been suggested that the carnitine is involved in the transportation of long-chain fatty acids into the mitochondria of those species that can metabolise them, and acetylcarnitine is thought to act as a ‘buffer’ for acetyl CoA (Brooks et al, 1974). In the spermatozoa of several species, intracellular concentrations of acetylcarnitine are closely linked to respiration, the rate of which declines as the concentration of acetylcarnitine decreases (Brooks, 1990). Since boar spermatozoa are unable to oxidize medium- or long-chain fatty acids yet can metabolize short-chain fatty acids (Jones and Chantrill, 1989), it is possible that acetylcarnitine constitutes a reserve energy source, but may also be present in some other unknown capacity. The inability of bromopyruvate to completely lower the ECPs during the first hour of incubation may be a consequence of the use of acetylcarnitine for the direct production of mitochondrial acetyl CoA which does not depend on stage 2 of the glycolytic pathway.

Triglycerides are known to be a metabolic energy source in spermatozoa of one species of sea urchin (Mita and Nakamura, 1993). Lipid globules within the mid-pieces of Glyptocidaris crenularis spermatozoa decreased in size when the cells were incubated in substrate-free sea water and there was an accumulation of free fatty acids. It was reported that there was some oxidation of fatty acids, although degradation of triglycerides in excess of fatty acid requirements would be unexpected. Boar spermatozoa mobilize glycerol from an unidentified source when incubated in the absence of an exogenous substrate (Jones and Milmlow, 1997) and the present study has shown that the amount of glycerol produced by saponification decreased rapidly early in the incubation. The source of the glycerol may be triglycerides, but as boar spermatozoa possess a pool of diglycerides that are actively involved in phospholipid synthesis (Vazquez and Roldan, 1997), this pool may also be responsible. Enzymatic estimation of triglycerides is not specific since the assay will also be positive with mono- and diglycerides. The production of glycerol by boar spermatozoa is accompanied by the
accumulation of long-chain fatty acids, primarily C_{16}-C_{22}, the amounts of which increase in the early stages of the incubation. As is the case with the apparent fatty acid over-production by sea urchin spermatozoa, it is possible that the di- or triglycerides contain some short-chain fatty acids which are metabolized after release, leaving the long-chain fatty acids.

Another source of endogenous substrates appears to be membrane phospholipids, the degradation of which would lead to the production of glycerol 3-phosphate which is rapidly metabolized (Jones and Gillan, 1996a,b). Although glycerol 3-phosphate could not be detected in boar spermatozoa incubated without exogenous substrates (Jones and Milmlow, 1997), the present study showed that incubations performed in the presence of (R,S)-α-bromohydryln phosphate, an inhibitor of the FAD-linked glycerol 3-phosphate dehydrogenase (Jones and Gillan, 1996a), produced measurable amounts of glycerol 3-phosphate which increased markedly after 5 h. The appearance of phosphorylcholine and phosphorylethanolamine only during the later stages of the incubation indicates an alternate breakdown of phospholipids to produce glycerol.

Glycerol 3-phosphate could also be produced from glycerylphosphorylcholine, a component of boar spermatozoa which is also present in boar semen (1.3%) (Jones, 1978). Glycerylphosphorylcholine is easily detectable in boar seminal plasma by NMR (Kamp and Lauterwein, 1995) and its presence in boar spermatozoa was confirmed in the present study. The amount of glycerylphosphorylcholine in boar spermatozoa remains constant for the first 4 h of incubation, after which it is rapidly and almost totally degraded, with the concomitant production of choline, accompanied by an increase in the concentration of glycerol 3-phosphate when the FAD-linked glycerol 3-phosphate dehydrogenase is inhibited. Whether this is a dedicated role for intracellular glycerol-phosphorylcholine is not known. Brooks et al. (1974) suggested that glycerolphosphorylcholine contributes to the total osmotic pressure of caudal epididymal fluid, so it may have a similar role within epididymal spermatozoa.

In the present study, acetate could be detected throughout the incubation period in amounts that increased markedly from about 5 h, but there was no indication of its source. Acetate can be produced from pyruvate by the spermatozoa of several species (Bedford and Hoskins, 1990) but it is unlikely that this mechanism was operating in the present study since the concentration of lactate (and presumably pyruvate) was extremely low.

Apart from the endogenous compounds in the present study (Fig. 4), there may be other endogenous compounds mobilized by boar spermatozoa that were depleted before the incubations had started. Cauda epididymides were collected from boars over a 1 h period, 20 min after the animals were killed, and were transported to the laboratory (1.5 h), and the spermatozoa were collected and washed (1 h) before the start of the incubations. During these 3–4 h, other substrates may have been used. However, the ability of the cells to maintain the capability to produce ATP extended beyond 10 h. Samples incubated aerobically at 34°C for up to 48 h in the absence of any exogenous substrate, although showing obvious signs of deterioration, still maintained high concentrations of ATP with ECPs above 0.6.

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Fig. 4. Approximate time course of mobilization of endogenous compounds by boar spermatozoa incubated in the absence of an exogenous substrate at 34°C over 10 h. A and B indicate the pathways inhibited by bromopyruvate (BPA) and (R,S)-α-bromohydryln phosphate (α-BHP), respectively.
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