Metabolism of glycerol 3-phosphate by mature boar spermatozoa

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Under anaerobic conditions boar spermatozoa metabolized fructose and glucose to lactate but did not produce ATP to the extent of that produced under aerobic conditions; the ketogenic amino acids leucine, tryptophan, phenylalanine and tyrosine were not oxidatively metabolized. Glycerol 3-phosphate was metabolized rapidly in the presence of or absence of the glycolytic inhibitor, 3-chloro-1-hydroxypropanone (CHOP). In the absence of CHOP, glycerol 3-phosphate was converted to CO₂, lactate, glucose 6-phosphate and fructose 6-phosphate, and ATP was produced. In the presence of CHOP, glycerol 3-phosphate did not produce CO₂, lactate or ATP, but formed fructose 1,6-bisphosphate and dihydroxyacetone phosphate. With dihydroxyacetone phosphate as substrate, fructose 1,6-bisphosphate, lactate, glucose 6-phosphate, fructose 6-phosphate and ATP were produced. Accumulation of glucose 6-phosphate and fructose 6-phosphate from glycerol 3-phosphate appeared to depend on the production of ATP; if ATP was not produced, dihydroxyacetone phosphate and fructose 1,6-bisphosphate accumulated. The conversion of glycerol 3-phosphate to glycolytic intermediates appeared to be a mechanism for the conversion of substrates for the ultimate production of lactate.

Introduction

"Two metabolic processes, namely glycolysis and respiration, stand out as prominent features of ejaculated semen; both are bound up with the activity of spermatozoa.” (Mann and Lutwak-Mann, 1981). However, it is apparent that there is a difference between species in the degree to which these metabolic processes are used. Under anaerobic conditions bull and ram spermatozoa, for example, utilize fructose and maintain high motility, which is accompanied by the production of lactate (Mann and Lutwak-Mann, 1981), whereas boar (Aalbers et al., 1961; Nevo et al., 1970) and guinea-pig (Frenkel et al., 1975) spermatozoa exhibit both decreased motility and fructose utilization, and do not produce lactate. Boar spermatozoa are extremely selective in the substrates that they oxidize; these comprise glucose, fructose, glycerol, glycerol 3-phosphate, lactate and, to a limited extent, acetate, suggesting that the glycolytic pathway is the major route for the production of mitochondrial substrates (Stevenson and Jones, 1982; Jones et al., 1992). The rapid oxidation of endogenous lactate and the extremely low degree of oxidation of pyruvate by these cells indicates that lactate is the major substrate for mitochondrial metabolism (Jones and Chantry, 1989).

Studies with the glycolytic inhibitor, 3-chloro-1-hydroxypropanone (CHOP), which at 0.5 mmol l⁻¹ inhibits boar sperm glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase by about 70% (Jones and Cooney, 1987) via its conversion to (S)-3-chlorolactaldehyde (Cooney and Jones, 1988), show that the metabolism of glycerol 3-phosphate by boar spermatozoa is unaffected by its presence.

Materials and Methods

Chemicals and substrates

D-[U-14C]glucose, D-[U-14C]fructose, [U-14C]glycerol, l-[U-14C]glycerol 3-phosphate ammonium salt, sodium l-[U-14C]lactate, sodium [U-14C]acetate, l-[U-14C]leucine, l-[U-14C]phenylalanine and l-[phenyl-U-14C]tryptophan were purchased from Amersham International plc (Amersham, Bucks). L-[Carboxyl-14C]tyrosine was supplied by Calbiochem (La Jolla, CA). Dihydroxyacetone phosphate (DHAP) was produced from its dimethyl ketal dicyclohexylamine salt according to the supplier’s instructions (Boehringer-Mannheim, Castle Hill, NSW). Enzymes, substrates and cofactors were obtained from Boehringer-Mannheim, while all other chemicals and reagents were of analytical grade and all solutions were prepared in water purified by reverse osmosis.

3-Chloro-1-hydroxypropanone (CHOP) was prepared from 3-chloro-1-hydroxypropanone dimethyl acetal by the procedure of Jones et al. (1986). Neutralized solutions of CHOP (10 mmol l⁻¹) were prepared immediately before use and added to incubations at a final concentration of 0.5 mmol l⁻¹.

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Collection and preparation of spermatozoa

Cauda epididymides, excised from the testis—epididymides complexes of mature boars (Sus scrofa: various crosses of Duroc, Hampshire, Landrace and Large White) 15–20 min after slaughter at the abattoir, were immediately placed in Dewar flasks containing PBS (Stevenson and Jones, 1985) at 34°C. The sealed flasks were transported to the laboratory where the spermatozoa were flushed with PBS at 34°C from incisions made essentially in the third and fourth segments of the cauda 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 to lower the content of endogenous substrates 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 about 106 cells ml−1 containing 15–25 mg protein ml−1. Separate ejaculates, collected from three Large White boars, were immediately centrifuged and the seminal plasma stored frozen until required.

Incubations with spermatozoa

Aerobic incubations were performed at 34°C in stoppered 25 ml Warburg flasks shaken at 120 cycles min−1 with air as the gas phase. Each incubate was composed of substrate (50 μl, 6 mmol l−1 with respect to its carbon content: 100–200 nCi if radioactive) and either PBS (50 μl) or CHOP (50 μl, 0.5 mmol l−1) and was begun by addition of the sperm suspension (900 μl, approximately 105 cells). Anaerobic incubations were carried out in 25 ml side-arm Warburg flasks through which nitrogen was passed for 2 min before the incubation was begun. Incubations were terminated at the appropriate times by addition of 100 μl 3 mol HClO4 l−1 and the amount of 14CO2 trapped as Na2CO3 was estimated (Dawson, 1977) according to established procedures (Stevenson and Jones, 1982). Time-course incubations were performed in open 25 ml conical flasks and aliquots (1 ml) were taken at specified periods and added to 100 μl 3 mol HClO4 l−1. Zero-time samples were removed approximately 10–15 s after preparation of the incubation suspensions. Washed caudal spermatozoa were suspended in PBS, disrupted by the addition of 3 mol HClO4 l−1, centrifuged (2000 g for 10 min), and the supernatant solution, together with sperm-free seminal fluid, assayed for glycerol 3-phosphate. Neutralized, deproteinized and desalted incubation solutions (Stevenson and Jones, 1982) were used for the assays of fructose (Beutler, 1984) fructose 1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Michal, 1984a), lactate and glycerol 3-phosphate (Lang, 1984), ATP (Jaworek and Welsch, 1985a), ADP and AMP (Jaworek and Welsch, 1985b), glucose 6-phosphate and fructose 6-phosphate (Michal, 1984b), using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto). The presence of CHOP in incubation solutions neither interfered with any of the assays nor was it a substrate for any of the enzymes used in the estimations. Protein was determined (Lowry et al., 1951) using BSA as a standard and the results were calculated using the linear transform equation of Coakley and James (1978). Energy charge potentials (ECPs) were calculated according to the formula of Atkinson and Walton (1967), where

\[
\text{ECP} = \left[\text{ATP}\right] + 0.5\left[\text{ADP}\right]/\left[\text{ATP}\right] + \left[\text{ADP}\right] + \left[\text{AMP}\right].
\]

Statistical analyses

Values are presented as means ± SEM for the number (n) of experiments performed.

Results

When incubated with a variety of substrates, boar spermatozoa oxidatively metabolized fructose, glucose, glycerol 3-phosphate, lactate and acetate, but not the ketogenic amino acids, leucine, tryptophan, phenylalanine or tyrosine (Table 1). Lactate accumulated from fructose, glucose, glycerol 3-phosphate and glycerol under aerobic conditions, but only from fructose and glucose when the incubation was carried out anaerobically. Energy charge potentials under anaerobic conditions with all substrates remained low (0.25–0.35).

With glycerol 3-phosphate as the substrate, the spermatozoa maintained a high ECP (0.7–0.77) but in the presence of 3-chloro-1-hydroxypropanone (CHOP) this rapidly declined over 15 min to a constant low value (0.10–0.17) over the remaining period of incubation (Fig. 1a). The increase in the concentration of lactate was constant for 1 h but there was no increase when CHOP was present (Fig. 1b).

In either the presence or absence of CHOP, the concentration of the substrate glycerol 3-phosphate declined at almost equivalent rates, decreasing by 85% after 30 min and by more than 94% after 1 h (Fig. 2a). The evolution of CO2 continued in an almost linear fashion over the 1 h incubation and for a further hour afterwards, when there was virtually no substrate present. Carbon dioxide was not evolved to any appreciable

<table>
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<tr>
<th>Substrate</th>
<th>CO2 (nmol mg−1 protein)</th>
<th>Aerobic</th>
<th>Anaerobic</th>
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<tr>
<td>Fructose</td>
<td>100 ± 2</td>
<td>26 ± 2</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>Glucose</td>
<td>96 ± 4</td>
<td>32 ± 5</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Glycerol 3-phosphate</td>
<td>25 ± 5</td>
<td>20 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>48 ± 3</td>
<td>23 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Lactate</td>
<td>71 ± 4</td>
<td>—</td>
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<tr>
<td>Acetate</td>
<td>22 ± 2</td>
<td>2</td>
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<tr>
<td>Leucine</td>
<td>0.03</td>
<td>2</td>
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<td>Tyrosine</td>
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Substrates were provided at a final concentration of 6 mmol l−1 with respect to carbon (n = 6–11).
Fig. 1. (a) Energy charge potential and (b) lactate production by boar spermatozoa incubated at 34°C with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) and absence (○) of 3-chloro-1-hydroxypropanone (CHOP) (0.5 mmol l⁻¹) (n = 5–6).

Fig. 2. (a) Concentration of glycerol 3-phosphate and (b) CO₂ production in boar spermatozoa incubated at 34°C with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) and absence (○) of 3-chloro-1-hydroxypropanone (CHOP) (0.5 mmol l⁻¹) (n = 12–15).

extent over the 2 h when CHOP was present in the incubation (Fig. 2b).

Fructose 1,6-bisphosphate and dihydroxyacetone phosphate accumulated when spermatozoa were incubated with glycerol 3-phosphate as the substrate in the presence of CHOP (Fig. 3a, b). However, after reaching maxima after 30–40 min, their concentrations declined over the remainder of the 2 h incubation. These glycolytic intermediates did not accumulate to any significant extent when CHOP was absent and there was little significant difference in the concentration of glycerol 3-phosphate whether CHOP was present or absent (Fig. 3c). Coinciding with the decline in the concentrations of fructose 1,6-bisphosphate and dihydroxyacetone phosphate was an accumulation of glucose 6-phosphate and fructose 6-phosphate in the absence of CHOP but, in its presence, the concentrations of these glycolytic intermediates increased by far smaller extents (Fig. 4).

With fructose (1 mmol l⁻¹) as substrate, lactate accumulated rapidly as the concentration of substrate declined (Fig. 5a). However, in the presence of CHOP, only 50% of the fructose was metabolized and the lactate concentration remained low (Fig. 5b). There was no detectable production of glucose 6-phosphate or fructose 6-phosphate in the presence or absence of CHOP, even though CHOP caused the accumulation of fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glycerol 3-phosphate (Jones et al., 1986).

Incubations with DHAP as the substrate maintained a high ECP (0.7–0.8) over the 2 h incubation. The concentration of DHAP declined, while those of fructose 1,6-bisphosphate, lactate, glucose 6-phosphate and fructose 6-phosphate increased (Fig. 6).

Discussion

Boar spermatozoa use a limited number of substrates for the generation of ATP in vitro. Fructose, glucose, glycerol, glycerol 3-phosphate and lactate are metabolized, whereas exogenous
pyruvate is oxidized only to a limited extent (Jones and Chantrill, 1989; Jones et al., 1992). Medium- and short-chain fatty acids, apart from acetate (Jones and Chantrill, 1989), are not oxidized and the present study has shown that the ketogenic amino acids cannot be used as precursors of acetyl CoA. Under aerobic conditions, high ECPs can be maintained by boar spermatozoa with glucose and fructose (Stevenson and Jones, 1982) and with glycerol and glycerol 3-phosphate (Jones et al., 1992) as substrates, and in the study reported here there was an accumulation of lactate to a concentration of 20–30 mmol mg protein⁻¹ from the trioses but to much higher concentrations from the hexoses. Under anaerobic conditions, however, the ECP is not maintained; lactate accumulates from fructose and glucose but not from glycerol and glycerol 3-phosphate, owing to the inability of the cells to regenerate oxidized cofactors. These observations, together with the fact that lactate is oxidized at a far greater rate than that of pyruvate (Jones and Chantrill, 1989), suggest that boar spermatozoa are reliant on the glycolytic pathway primarily to produce lactate, rather than ATP, and that it is lactate that is the main metabolic fuel for the mitochondrial synthesis of ATP. A similar conclusion has been reached by Calvin and Tubbs (1978), who treated boar spermatozoa hypotonically to remove the plasma membrane, which were then demonstrated to oxidize endogenous NADH by such a lactate–pyruvate shuttle.

The studies reported here on the metabolism of glycerol 3-phosphate confirmed that boar spermatozoa produce lactate from this substrate but not at the same rate as its rate of consumption. The rapid decrease in the concentration of glycerol 3-phosphate, accompanied by the maintenance of a high ECP and the steady production of CO₂ for at least 2 h, suggests that the carbon atoms of the substrate are retained by the cells as intermediates for the production of lactate. Lactate and CO₂ account for approximately 57% of the carbon atoms after 1 h, and 85% after 2 h. Slight increases in the concentrations of DHAP and fructose 1,6-bisphosphate over the first hour, followed by substantial rises in the concentrations of glucose 6-phosphate and fructose 6-phosphate during the second hour, indicate that the cells conserve the carbons as potential lactate-producing substrates. In fact, glucose

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**Fig. 3.** Concentration of (a) fructose 1,6-bisphosphate, (b) dihydroxyacetone phosphate and (c) glyceraldehyde 3-phosphate in boar spermatozoa incubated at 34°C with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) and absence (○) of 3-chloro-1-hydroxypropanone (CHOP) (0.5 mmol l⁻¹) (n = 6).

**Fig. 4.** Concentration of (a) glucose 6-phosphate and (b) fructose 6-phosphate in boar spermatozoa incubated at 34°C with glycerol 3-phosphate (2 mmol l⁻¹) in the presence (●) and absence (○) of 3-chloro-1-hydroxypropanone (CHOP) (0.5 mmol l⁻¹) (n = 7).
Fig. 5. Concentration of fructose (●) and lactate (○) in boar spermatozoa incubated at 34°C with fructose (1 mmol l⁻¹) in the (a) absence and (b) presence of 3-chloro-1-hydroxypropanone (CHOP) (0.5 mmol l⁻¹) (n = 4).

Fig. 6. Concentration of (a) glyceraldehyde 3-phosphate (○) and dihydroxyacetone phosphate (●) (n = 10), (b) glucose 6-phosphate (○) and fructose 6-phosphate (●) (n = 5–6), (c) fructose 1,6-bisphosphate (n = 9) and (d) lactate (n = 6) in boar spermatozoa incubated at 34°C with dihydroxyacetone phosphate (2 mmol l⁻¹).

6-phosphate and fructose 6-phosphate accumulated in the proportions of 2:3:1 after 2 h. The production of these isomers from fructose 1,6-bisphosphate has been reported when boar spermatozoa were incubated with fructose 1,6-bisphosphate as the substrate in the presence of (S)-α-chlorohydrin (Jones and Montague, 1991). In that study, the equilibrium ratio of
glucose 6-phosphate:fructose 6-phosphate was determined as 3.1:1 at pH 7.4, in close agreement with 3.3:1, the ratio found with the pure enzyme determined at pH 8 (Kahana et al., 1960). Thus, it appears that boar spermatozoa can conserve the carbons of glycerol 3-phosphate as glycolytic intermediates.

In a study of the oxidation of glycerol 3-phosphate by rat caudal spermatozoa, Ford (1981) described a similar phenomenon. At a concentration of 10 mmol 1−1, glycerol 3-phosphate is oxidized rapidly but, as only 45% is accounted for as DHAP, lactate and CO₂, Ford (1981) suggested the formation of an 'unknown product'. Furthermore, as 'the observed oxygen uptake ... [was] less than or equal to the quantity of oxygen required to convert glycerol 3-phosphate to dihydroxyacetone phosphate and carbon dioxide ... the unknown product is unlikely to be more oxidized than dihydroxyacetone phosphate' (Ford, 1981). We suggest that the 'unknown product' in rat caudal spermatozoa is likely to be either fructose 1,6-biphosphate or glucose 6-phosphate and fructose 6-phosphate, or all three.

Use of CHOP, an inhibitor of boar spermatozoa glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase (Jones and Cooney, 1987), enabled the pattern of accumulation of glycolytic intermediates produced from glycerol 3-phosphate to be examined. In the presence of CHOP, the production of CO₂ and the accumulation of lactate was prevented; the ECP decreased but there was little or no effect on the consumption of glycerol 3-phosphate. The concentrations of DHAP and fructose 1,6-biphosphate increased markedly over 40 min and their subsequent decline was accompanied by the accumulation of only small amounts of glucose 6-phosphate and fructose 6-phosphate.

These results present an apparent anomaly. Why does a high concentration of fructose 1,6-biphosphate not lead to the production of glucose 6-phosphate and fructose 6-phosphate, yet, when the isomeric hexose phosphates do accumulate, there is not a marked increase in the concentration of fructose 1,6-biphosphate? It appears that ATP may in some way be necessary to establish the build-up of the hexose phosphates since this occurs only under conditions in which substrates can proceed beyond glyceraldehyde 3-phosphate to produce lactate; if ATP synthesis is prevented, fructose 1,6-biphosphate and dihydroxyacetone phosphate accumulate. Confirmation of the possible involvement of ATP in determining which glycolytic intermediates accumulate was obtained from two separate experiments. Spermatozoa incubated with fructose as the substrate produced a high concentration of lactate, there was a depletion of the substrate within about 1 h and no accumulation of the hexose phosphates. Identical incubations performed in the presence of CHOP, which prevents the production of ATP, did not produce lactate, did not produce the hexose phosphates and the consumption of fructose ceased after 10 min. The second experiment, in which DHAP was used as the substrate, produced high concentrations of lactate, fructose 1,6-biphosphate, glucose 6-phosphate and fructose 6-phosphate. Thus, it appears that substrates in excess of normal ATP-producing requirements entering the glycolytic pathway at the stage of triosephosphate formation (glycerol, glycerol 3-phosphate, fructose 1,6-biphosphate and DHAP), but not from hexoses (fructose and glucose), can be actively retained within the pathway for subsequent oxidation.

The rapid oxidation of glycerol 3-phosphate by boar spermatozoa leads to two questions: is there some physiological role for the ability of boar spermatozoa, and that of other species, to acquire and use glycerol 3-phosphate as a metabolic substrate and, if so, what is the source of this substrate? Glycerol 3-phosphate is oxidized readily in vitro by mature cock (Yamada and Terada, 1974), bull (Mohri and Masaki, 1967), ram (Mann and White, 1957), guinea-pig (Frenkel et al., 1975) and boar (present study) spermatozoa. However, it is evidently not used by rabbits (Keyhani and Storey, 1973), humans, dogs or stallions (Schenkman et al., 1965), as it fails to stimulate respiration in the mature cells of these species in vitro. As Ford (1981) has observed, glycerol 3-phosphate carries a strong negative charge and would not be expected to diffuse across cell membranes unless transported by a specific carrier. Evidence for the possible existence of such a carrier is that the plasma membrane of bovine spermatozoa has been shown to contain phosphate and succinate exchangers similar to those found in the mitochondrial membrane of other cells (Babcock et al., 1975), suggesting that the plasma membrane of spermatozoa may contain specific, and as yet unidentified, carriers for a number of biological compounds.

An alternative explanation for the use of glycerol 3-phosphate could be that it is not taken up per se but undergoes hydrolysis, or is susceptible to phosphatase activity, which converts it to glycerol (Jones and Porter, 1995). This is not the case with boar spermatozoa, since the build-up of glycolytic intermediates from glycerol 3-phosphate and glycerol showed completely different patterns when incubations were performed with both substrates in the presence of CHOP (Jones and Cooney, 1987).

The ability of spermatozoa to metabolize glycerol 3-phosphate in vitro indicates that this is a potential substrate in vivo, but the origin of this substrate in vivo is not known. While it was not detectable in caudal spermatozoa or in ejaculated semen, it is rapidly metabolized and would not, therefore, be expected to be present. Another possibility is that it is derived from phospholipids or, more specifically, glycerolphosphorylcholine (GPC). Although the addition of phospholipids ‘greatly prolonged the motility of [bull] spermatozoa in a buffered medium’ (Lardy and Phillips, 1941) and sea urchin spermatozoa can hydrolyse endogenous phospholipids to glycerol 3-phosphate (Mita and Ueta, 1990), attention has been focused on the function of GPC, first suggested as a potential source of energy for spermatozoa by Dawson et al. (1957). Glycerolphosphorylcholine is present in the epididymal plasma of many mammals, including African elephants (Darin-Bennett et al., 1976), humans (Sane et al., 1982), stallions, rams, dogs, rabbits, guinea-pigs, rats, hamsters and monkeys (1–3%), and boars have a concentration of 3.3% (Jones, 1978), but it cannot be used directly as an energy source. It is thought that a GPC diesterase, present in the uterine secretions of ewes, cows, sows and rats, hydrolyses GPC to glycerol 3-phosphate (White et al., 1963), while similar activity has been demonstrated within rat cauda epididymal cells (Bjerve and Reitan, 1978). Whatever the source of the compound, boar spermatozoa are particularly adept at using glycerol 3-phosphate as a substrate either for the direct production of ATP via lactate or for the production of
glycolytic intermediates for the potential production of ATP via lactate.

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