Effects of $\alpha$-chlorohydrin on the metabolism of testicular and epididymal spermatozoa of rams

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Summary. Spermatozoa were collected from the rete testis and vas deferens of conscious rams. The endogenous oxygen uptake of the spermatozoa was unaffected by $\alpha$-chlorohydrin added in vitro, although this compound abolished the stimulation of oxygen uptake caused by the addition of glycerol. The metabolism of $[14C]$glycerol by testicular and epididymal spermatozoa was markedly reduced by $\alpha$-chlorohydrin, CO$_2$ production and lactate accumulation being almost totally inhibited. These effects were dependent upon a period of preincubation of the spermatozoa with $\alpha$-chlorohydrin alone, since the presence of glycerol protected the spermatozoa from its action. Longer exposure and a higher concentration of $\alpha$-chlorohydrin were needed with testicular than with epididymal spermatozoa to achieve a maximal effect. The metabolism of $[14C]$glucose by both sperm types was also inhibited by $\alpha$-chlorohydrin. Spermatozoa of the ram are therefore susceptible to the action of $\alpha$-chlorohydrin throughout the epididymis, although more mature spermatozoa are more affected. It is suggested that $\alpha$-chlorohydrin is converted to an intermediate which is the agent responsible for the inhibition of glycolysis in spermatozoa.

Introduction

The antifertility agent $\alpha$-chlorohydrin has been shown to act post-testicularly to cause a reversible loss of fertility in several species of mammals (Coppola, 1969; Ericsson & Baker, 1970; Lubicz-Nawrocki & Chang, 1974). Although $\alpha$-chlorohydrin is unlikely ever to be an acceptable antifertility compound in man, because of its high toxicity in primates (Setty, Kar, Roy & Chowdhury, 1970), the elucidation of its mode and site of action in the epididymis is clearly important. If the primary site of action is on the spermatozoa themselves, then a specific metabolic site might be found which could be vulnerable to attack by other compounds, or by active intermediates of $\alpha$-chlorohydrin with lower toxicity in primates.

It has been shown that $\alpha$-chlorohydrin is active in the ram (Kreider & Dutt, 1969), and metabolic studies have shown that the glycolytic pathway in ejaculated ram spermatozoa is inhibited by $\alpha$-chlorohydrin administered in vivo and in vitro (Mohri, Suter, Brown-Woodman, White & Ridley, 1975). Because it is possible to collect large quantities of spermatozoa from the rete testis and vas deferens of the ram using cannulation techniques, the nature of the effect of $\alpha$-chlorohydrin on the metabolism of spermatozoa can be investigated, and it can be determined whether the spermatozoa are as susceptible when freshly released from the germinal epithelium as they are after storage in the cauda epididymidis.

Materials and Methods

Sperm samples. Testicular spermatozoa were obtained in rete testis fluid collected from four conscious Clun Forest rams. The extratubular rete was cannulated as described previously (Voglmayr, Waites & Setchell, 1966; Voglmayr, Scott, Setchell & Waites, 1967) and the rete testis fluid was drawn along the cannula and into a vessel cooled to 4°C. Samples were removed after 12–18 hr collection.

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The catheters remained patent for up to 14 days. The vas deferens was also cannulated with a polythene cannula (1·0 mm i.d., 1·4 mm o.d.) and the contents of the cauda epididymidis allowed to move slowly into a cooled syringe from which samples were collected periodically over a period of about 4 days (Text-fig. 1). Epididymal spermatozoa were collected from two Suffolk rams by cannulation of the vas deferens.

**Sample preparation.** Testicular spermatozoa were incubated in rete testis fluid for measurement of oxygen uptake. In other experiments, testicular spermatozoa were recovered from rete testis fluid by low-speed (1000 g) centrifugation for 10 min, and resuspended in phosphate-buffered saline (PBS) containing 100 mM-NaCl, 3 mM-KCl, 6·5 mM-Na2HPO4, 1·5 mM-KH2PO4, 0·8 mM-CaCl2 and 0·5 mM-MgCl2. Epididymal spermatozoa were washed by being suspended in PBS, recovered by centrifugation and resuspended for incubation. Sodium benzyl penicillin (1·3 mg/ml) and streptomycin sulphate (1 mg/ml) were added to the incubation media. The sperm concentration in all incubations was of the order of 1 × 10⁸/ml.

**Oxygen uptake of spermatozoa.** Rete testis fluid and epididymal spermatozoa, suspended in PBS, were incubated at 34°C in a ‘Clark’-type oxygen electrode (Rank Brothers, Bottisham, Cambs). The electrode was calibrated by the method of Robinson & Cooper (1970). The endogenous oxygen uptake of the cells with or without addition of α-chlorohydrin was recorded for 30 min. Substrate was then added and the oxygen uptake again monitored.

**Carbon dioxide production.** Rete testis fluid or testicular and epididymal spermatozoa suspended in PBS were incubated at 34°C in 25-ml conical flasks in a Dubonoff incubator. The flasks were fitted with ‘Suba-seal’ stoppers and glass hanging wells. After a period of preincubation with or without added α-chlorohydrin, [U-14C]glycerol or [U-14C]glucose was added. Ten minutes before the end of the incubation, 0·25 ml hyamine hydroxide (1·0 M in methanol) was injected into the hanging wells. The incubation was terminated by the injection of 0·1 ml 2 M-H2SO4 into the flasks which then remained stoppered for a further 30 min. The hanging wells were removed, wiped and placed in scintillation vials. Scintillation fluid was added and the radioactivity in the trapped CO₂ was estimated.

**Incorporation of substrates into lipids.** After incubation for the trapping of ¹⁴CO₂ (see above) the incubation media were centrifuged and the supernatants removed. The pellets of spermatozoa were extracted three times with chloroform:methanol (2:1, v/v) and washed three times with 0·37 %
(w/v) KCl. The extracted lipid was taken to dryness under N₂ in scintillation vials. Scintillation fluid was added and the radioactivity present in the lipids was estimated.

Lactate production. Following incubations for the trapping of ¹⁴CO₂ (see above) the incubation media were centrifuged and the supernatant removed and stored at −12°C. The lactate content of these supernatants was subsequently estimated enzymatically by the method of Hohorst (1963).

Scintillation counting. The samples were counted in toluene:triton-X100 (9:1, v/v) containing 2.0 g PPO and 0.25 g POPOP per litre. The radioactivity was estimated in a liquid scintillation spectrometer (Nuclear Enterprises Ltd, NE8310, Edinburgh, Scotland) and corrected to 100% by use of an external standard quench correction.

Chemicals. [U-¹⁴C]Glycerol and [U-¹⁴C]glucose were supplied by the Radiochemical Centre, Amersham, Bucks. 3-Chloro-1,2 propanediol (α-chlorohydrin) was obtained from B.D.H. Chemicals Ltd, Poole, Dorset, and purified by distillation under reduced pressure. Lactate dehydrogenase, and NAD⁺ were purchased from Boehringer (London) Ltd.

Results

Oxygen uptake

In control incubations the addition of 0.4 mm-glycerol caused a 36 ± 11% (mean ± S.E.M., n (no. of observations) = 6) increase in the oxygen uptake of testicular spermatozoa in rete testis fluid and a 40 ± 7% (n = 7) increase in the oxygen uptake of epididymal spermatozoa suspended in PBS. Following incubation for 30 min with 1.27 mm-α-chlorohydrin, however, there was no increase in oxygen uptake. The addition of 1.27 mm-α-chlorohydrin alone to testicular spermatozoa in rete testis fluid, or to epididymal spermatozoa in PBS did not alter the endogenous oxygen uptake of these cells.

Metabolism of [¹⁴C]glycerol

When testicular and epididymal spermatozoa were preincubated for 30 min with 1 mm-α-chlorohydrin, their subsequent metabolism of [¹⁴C]glycerol was markedly reduced (Table 1). The production of ¹⁴CO₂ was almost totally inhibited, as was the accumulation of lactate in the incubation medium. The incorporation of ¹⁴C from [¹⁴C]glycerol into the lipids of testicular and epididymal spermatozoa was reduced to 40 to 50% of control values. Subsequent experiments showed that this effect upon the incorporation of radioactivity into lipids was variable and was only observed when control levels of incorporation were high.

Effect of preincubation with α-chlorohydrin. Testicular and epididymal spermatozoa were preincubated for various times with 1 mm-α-chlorohydrin before the addition of 2 mm-[¹⁴C]glycerol to the incubation flasks. If α-chlorohydrin was added at the same time as [¹⁴C]glycerol, the production of ¹⁴CO₂ by either sperm type during 1 hr was not inhibited (Text-fig. 2). Preincubation of epididymal

![Text-fig. 2](image-url)
Table 1. The effect of 1 mm-α-chlorohydrin on the metabolism of 2 mm-(1 µCi) [14C]glycerol by ram spermatozoa (means of two determinations expressed as nmol/10^8 spermatozoa at 34°C)

<table>
<thead>
<tr>
<th>Period of incubation (min)</th>
<th>Glycerol converted to CO₂</th>
<th>Glycerol converted to lipid</th>
<th>Lactate accumulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>α-Chlorohydrin</td>
<td>Control</td>
</tr>
<tr>
<td>Epididymal spermatozoa</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>6.3</td>
<td>0.3</td>
<td>0.5</td>
</tr>
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<td>30</td>
<td>64</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>60</td>
<td>148</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>120</td>
<td>288</td>
<td>1.2</td>
<td>2.5</td>
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<tr>
<td>180</td>
<td>548</td>
<td>2.4</td>
<td>2.2</td>
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<td>Testicular spermatozoa</td>
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</tr>
<tr>
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<td>0.5</td>
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<td>30</td>
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</table>

spermatozoa with 1 mm-α-chlorohydrin for 5 min was sufficient to obtain a maximal inhibition of 14CO₂ production and lactate accumulation. However, the preincubation time needed to achieve maximal inhibition of 14CO₂ production by testicular spermatozoa was 30 min.

**Effect of α-chlorohydrin concentration.** Preincubation for 30 min with a threshold concentration of 0.1 mm-α-chlorohydrin was sufficient to produce a maximal inhibition of 14CO₂ production from [14C]glycerol by epididymal spermatozoa (Text-fig. 3). With testicular spermatozoa in PBS or rete testis fluid maximal inhibition of 14CO₂ production and lactate accumulation was produced by preincubation with concentrations of 0.2-0.5 mm-α-chlorohydrin.

Text-fig. 3. The effect of α-chlorohydrin concentration on (a) CO₂ produced, and (b) lactate accumulated from the metabolism of 2 mm-glycerol by ram spermatozoa from the testis (■, in rete testis fluid; △, in phosphate buffer) and cauda epididymidis (○, in PBS). Each point is the mean of 4 determinations.

**Metabolism of [14C]glucose**

When testicular and epididymal spermatozoa were preincubated for 30 min with 1 mm-α-chlorohydrin and subsequently incubated for 1 hr with [14C]glucose, there was an inhibition of 14CO₂ production, 14C incorporation into lipids and lactate accumulation. This inhibition was similar to that observed with [14C]glycerol as substrate (Table 2).
**Discussion**

It has been shown that α-chlorohydrin inhibits the in-vitro glycolytic metabolism of testicular and epididymal spermatozoa of the ram. The presence of glycerol apparently protects spermatozoa from this damage, which suggests that some derivative of α-chlorohydrin is formed by spermatozoa and is the active agent in the inhibition of glycolysis. These findings agree with those of Mohri et al. (1975), who have demonstrated the in-vitro inhibition of glycolysis in washed ejaculated ram spermatozoa incubated with 0.1 mM-α-chlorohydrin, and have suggested that this inhibition is due to the formation of a phosphorylated intermediate of α-chlorohydrin, which in some way reduces the activity of the enzymes glyceraldehyde 3-phosphate dehydrogenase and triose-phosphate isomerase in spermatozoa. The protective effect of glycerol may reflect competition of this compound with α-chlorohydrin for a transport system or metabolic pathway leading to the formation of this active intermediate. The present study has shown that the inhibition of glycolysis is not confined to ejaculated spermatozoa but probably occurs in spermatozoa throughout the epididymis.

In earlier experiments (Edwards, Jones & Waites, 1975), no consistent effect of α-chlorohydrin on the incorporation in vivo of [14C]glycerol into lipids in the epididymides of rats could be demonstrated. In the present experiments, the effect of α-chlorohydrin upon the incorporation of [14C]glycerol into ram sperm lipids in vitro was extremely variable and far less marked than the effect of this compound upon glycolysis in these cells.

To achieve maximal inhibition of glycolysis, testicular spermatozoa require a longer period of preincubation and exposure to a higher concentration of α-chlorohydrin than epididymal spermatozoa. The difference may be due to altered cell permeability to α-chlorohydrin as the spermatozoa mature, or it may reflect a more active enzymatic transformation of the compound by the more mature cells. It is likely therefore that higher doses of the compound, or lower doses given to animals for a longer period of time, inhibit glycolysis not only in spermatozoa in the cauda epididymidis but also in those entering the caput epididymidis. This would result in a longer period of infertility following the withdrawal of the drug. Such an effect has been observed in rats, in which the time taken to return to normal fertility after giving α-chlorohydrin was dose-dependent (Vickery, Erickson & Bennett, 1974).

It appears that α-chlorohydrin exerts its antifertility action on spermatozoa by arresting glycolysis, without any marked effect on endogenous oxygen uptake. Presumably this inhibition of glycolysis causes infertility by reducing the metabolism of spermatozoa after ejaculation, preventing their utilization of seminal fructose. Many workers have commented on the relatively low motility of spermatozoa in samples of semen and epididymidal contents taken from treated animals (Coppola, 1969; Samojlik & Chang, 1970; Johnson & Pursel, 1973; Brown & White, 1973), and Vickery et al. (1974) showed that spermatozoa taken from male rats made infertile with minimal doses (2.5 mg/kg) of α-chlorohydrin are able to fertilize ova if they are transferred directly into the ovarian bursa. However, other workers have concluded that the major effect of α-chlorohydrin is upon the fertilizing
ability of spermatozoa rather than upon their motility (Tsunoda & Chang, 1976). The relationship between impaired glycolysis of spermatozoa and their reduced fertility remains to be elucidated.

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References


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