Mode of Action of α -Chlorohydrin as a Male Anti-Fertility Agent

INHIBITION OF THE METABOLISM OF RAM SPERMATOZOA BY α -CHLOROHYDRIN AND LOCATION OF BLOCK IN GLYCOLYSIS

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1. The effect of α -chlorohydrin on the metabolism of glycolytic and tricarboxylate-cycle substrates by ram spermatozoa was investigated. The utilization and oxidation of fructose and triose phosphate were much more sensitive to inhibition by α -chlorohydrin (0.1–1.0 mM) than lactate or pyruvate. Inhibition of glycolysis by α -chlorohydrin is concluded to be between triose phosphate and pyruvate formation. Oxidation of glycerol was not as severely inhibited as that of the triose phosphate. This unexpected finding can be explained in terms of competition between glycerol and α -chlorohydrin. A second, much less sensitive site, of α -chlorohydrin inhibition appears to be associated with production of acetyl-CoA from exogenous and endogenous fatty acids. 2. Measurement of the glycolytic intermediates after incubation of spermatozoal suspensions with 15 mM-fructose in the presence of 3 mM- α -chlorohydrin showed a 'block' in the conversion of glyceraldehyde 3-phosphate into 3-phosphoglycerate. α -Chlorohydrin also caused conversion of most of the ATP in spermatozoa into AMP. After incubation with 3 mM- α -chlorohydrin, glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase activities were decreased by approx. 90% and 80% respectively, and in some experiments aldolase was also inhibited. Other glycolytic enzymes were not affected by a low concentration (0.3 mM) of α -chlorohydrin. Loss of motility of spermatozoa paralleled the decrease in glyceraldehyde 3-phosphate dehydrogenase activity. α -Chlorohydrin, however, did not inhibit glyceraldehyde 3-phosphate dehydrogenase or triose phosphate isomerase in sonicated enzyme preparations when added to the assay cuvette. 3. Measurement of intermediates and glycolytic enzymes in ejaculated spermatozoa before, during and after injection of rams with α -chlorohydrin (25 mg/kg body wt.) confirmed a severe block in glycolysis *in vivo* at the site of triose phosphate conversion into 3-phosphoglycerate within 24 h of the first injection. Glyceraldehyde 3-phosphate dehydrogenase activity was no longer detectable and both aldolase and triose phosphate isomerase were severely inhibited. Spermatozoal ATP decreased by 92% at this time, being quantitatively converted into AMP. At 1 month after injection of α -chlorohydrin glycolytic intermediate concentrations returned to normal in the spermatozoa but ATP was still only 38% of the pre-injection concentration. Motility of spermatozoa was, however, as good as during the pre-injection period. The activity of the inhibited enzymes also returned to normal during the recovery period and 26 days after injection were close to pre-injection values. 4. An unknown metabolic product of α -chlorohydrin is suggested to inhibit glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase of spermatozoa. This results in a lower ATP content, motility and fertility of the spermatozoa. Glycidol was shown not to be an active intermediate of α -chlorohydrin *in vitro*.

α -Chlorohydrin (3-chloropropane-1,2-diol) has been shown to cause reversible infertility in the rat, guinea pig, ram and boar when administered orally or by injection (Coppola, 1969; Ericsson & Baker, 1970; Kreider & Dutt, 1970; Brown-Woodman *et al.*, 1975). At low doses it does not affect libido (Ericsson, 1970) and the site of action appears to be

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on spermatozoa in the cauda epididymis rather than in the testis (Samojlik & Chang, 1970; Crabo & Appelgren, 1972; Brown & White, 1973; Brown-Woodman & White, 1974, 1975). Such a compound has advantages over drugs inhibiting spermatogenesis in the testis, since the delay in the onset of infertility is not so long and there is less possibility of genetic damage.

Clearly α -chlorohydrin has many of the desirable features of a male contraceptive. Toxic side effects in

animals, particularly the monkey (Setty *et al.*, 1970; Brown-Woodman & White, 1975), have, however, prevented fertility trials of α -chlorohydrin in man, and it is important therefore to elucidate the mode of action so that a rational approach may be made to the synthesis of less toxic analogues. Apart from its contraceptive possibilities, α -chlorohydrin is also a useful experimental tool for studying the biochemistry of the epididymal environment and its interaction with spermatozoa.

Previous work in our laboratory has shown that the fertility of rams is temporarily decreased within 2 days of injecting α -chlorohydrin and that this is mirrored by decreased metabolic activity of the ejaculated spermatozoa, which may account for the anti-fertility action of the drug (Brown & White, 1973; Brown-Woodman *et al.*, 1974). We have also demonstrated that α -chlorohydrin severely inhibits the O_2 consumption and aerobic utilization of glucose by washed ram spermatozoa *in vitro* (Brown-Woodman *et al.*, 1975; Suter *et al.*, 1975).

The work in the present paper, some of which has been summarized by Mohri *et al.* (1975), is concerned with pin-pointing the site of action of α -chlorohydrin in spermatozoa.

Materials and Methods

Semen and washing of spermatozoa

Ram semen was collected by electrical stimulation (Blackshaw, 1954). Ejaculates were pooled where necessary, diluted with 4 vol. of Ca^{2+} -free Krebs-Ringer phosphate medium (Umbreit *et al.*, 1964b) and centrifuged at 750g (r_{av} , 18cm) for 10 min at 20°C. The supernatant was removed and the sedimented spermatozoa were resuspended in the Krebs-Ringer phosphate medium, centrifuged again at 750g (r_{av} , 18cm) for 10 min and the spermatozoa (2×10^8) finally suspended in 2 ml of the same medium for metabolic studies or in 10 ml of 0.25M-sucrose containing 1 mM-EDTA and 1 mM-2-mercaptoethanol at 0°C to give appropriate spermatozoa concentrations for sonication.

Spermatozoa counts

The spermatozoan suspension was diluted with formal saline (1% formalin in 0.9% NaCl) or with 0.9% NaCl to which 20% (w/v) $HClO_4$ was added. Samples of this suspension were transferred to a haemocytometer, left to settle for 5 min, and the spermatozoa were counted under a microscope.

Metabolic studies

The washed spermatozoa (approx. 2×10^8 /3 ml flask) were incubated for 3 h at 37°C in Warburg flasks (capacity 17 ml) in the presence of α -chlorohydrin (0, 0.1, 1, 10 and 100 mM) with the fol-

lowing substrates (0.5 μ Ci): [U - ^{14}C]fructose (5 mM), [U - ^{14}C]glycerol (5, 10, 20, 50, 100 mM), [U - ^{14}C]lactate (5 mM), [U - ^{14}C]pyruvate (10 mM), [U - ^{14}C]acetate (10 mM) and [U - ^{14}C]palmitate (2 mM) attached to 3% defatted bovine serum albumin (defatted by the method of Chen, 1967). In addition, endogenous respiration and oxidation of glycerol 3-phosphate (10 mM), dihydroxyacetone (10 mM) and dihydroxyacetone phosphate (5 mM) were investigated (see Tables 1–6 and Fig. 1).

O_2 consumption was measured by conventional Warburg techniques (Umbreit *et al.*, 1964a). The centre well contained 0.2 ml of CO_2 -free 2M-KOH and the side arm 0.2 ml of 2M-HCl. The flasks were shaken at 60 cycles/min with air as gas phase. The incubations were terminated by adding HCl from the side arms to liberate CO_2 formed during the experiment and the flasks shaken for a further 15 min (or until cessation of pressure changes). $HClO_4$ (0.2 ml of 30%) was then added to the flasks, which were left to extract on ice for 30 min; the mixture was then centrifuged at 39100g (r_{av} , 10 cm) for 10 min at 0°C. A measured sample of the supernatant was neutralized with KOH in the presence of 2 drops of Universal indicator (BDH, Poole, Dorset, U.K.) and the precipitate of $KClO_4$ removed by centrifugation [27000g (r_{av} , 10 cm) for 10 min]. The supernatant was diluted to a known volume and stored at 0°C until required for analysis.

The KOH from the centre well was quantitatively transferred to a graduated test tube and made up to 2.0 ml; CO_2 release was measured manometrically on a portion (0.5 ml) after the addition of 0.2 ml of 1M- H_2SO_4 . The radioactivity (d.p.m.) was determined in a sample (0.2 ml) in a Nuclear-Chicago liquid-scintillation counter; all counts were corrected for background and counting efficiency. The scintillation fluid consisted of 0.4% (w/v) 2,5-diphenyloxazole (PPO), 0.01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) (from Packard Instrument Co., IL 60515, U.S.A.) in toluene/Triton X-100 (2:1, v/v).

Fructose, lactate, glycerol and pyruvate were assayed by following the A_{340} change corresponding to the oxidation or reduction of nicotinamide nucleotides. Fructose (method of Klotzsch & Bergmeyer, 1963), glycerol (method of Wieland, 1974) and L-(+)-lactate (method of Hohorst, 1963) were measured spectrophotometrically in a Hitachi-Perkin-Elmer spectrophotometer. Pyruvate was measured by fluorimetry with a Farrand spectrofluorimeter by a modification (Suter, 1973) of the method of Bücher *et al.* (1963).

Assays of glycolytic intermediates

Suspensions of washed spermatozoa in Krebs-Ringer phosphate medium contained penicillin and

streptomycin (0.5 mg of each/ml) in a final volume of 3 ml and were incubated with fructose (15 mM) as substrate at 37°C for 3 h with constant shaking (see Tables 7 and 11). At the end of the incubation, 0.5 ml of 30% (w/v) HClO_4 was added to the suspensions and after removal of the deproteinized material by centrifugation [48000g (r_{av} , 10 cm) for 15 min at 4°C] the acid extract was neutralized to pH 5–6 with 5M- K_2CO_3 by using Universal indicator. The precipitated KClO_4 was removed by centrifugation [27000g (r_{av} , 10 cm) for 10 min] and the supernatant used for the assay of glycolytic intermediates and other substances. For the experiments, the results of which are shown in Tables 10 and 11, samples (usually 1–2 ml) of semen were centrifuged at 43000g (r_{av} , 10 cm) at 15°C for 15 min, the seminal plasma was removed and the spermatozoal pellet thoroughly extracted in ice-cold 6% HClO_4 (2.5 ml) with the aid of a glass stirring-rod and sonication (3 $\times$ 30 s at 16–24 kHz in a Mullard sonicator). After centrifugation at 36000g (r_{av} , 10 cm) at 0°C (15 min) the supernatant (HClO_4 extract) was transferred to a 10 ml graduated stoppered tube and the sediment was re-extracted with a further 1.0 ml of 6% HClO_4 . After centrifugation, the combined HClO_4 extracts were neutralized to pH 6.0 with 5M- K_2CO_3 and diluted to a known volume with water (usually 5 ml) before removal of precipitated KClO_4 by centrifugation [3200g (r_{av} , 18 cm) for 10 min at 4°C]. Adenine nucleotide assays were carried out on the extracts which were stored frozen (–20°C) as soon as practically possible (always within 24 h of semen collection) to minimize ATP loss. Assays of the remaining intermediates were performed within 4 days of semen collection.

Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate were determined by fluorimetry by using a Farrand spectrofluorimeter as described previously (Suter, 1973) by modification of spectrophotometric methods (Bergmeyer, 1963). Lactate, glycerol and adenine nucleotides (ATP, ADP and AMP), were measured spectrophotometrically with a Hitachi-Perkin-Elmer spectrophotometer by following the change in A_{340} corresponding to the oxidation or reduction of nicotinamide nucleotides (Bergmeyer, 1963). L-Glycerol 3-phosphate was determined spectrophotometrically by the method of Hohorst (1963).

Enzyme assays

Sonicated preparations (16–14 kHz for 5 min at 0°C) from spermatozoa were used as the enzyme source (see Tables 8, 9, 11). If necessary the original preparation was diluted with 0.25M-sucrose containing 1 mM-2-mercaptoethanol and 1 mM-EDTA to the desired concentrations. In some experiments, the

spermatozoal suspensions were incubated with fructose (15 mM) under the same conditions as described above for assays of glycolytic intermediates. After the incubation, the spermatozoa were centrifuged at 4°C, resuspended in the above medium and then sonicated as above. Hexokinase was assayed as described by Bergmeyer (1974a). Aldolase and glyceraldehyde 3-phosphate dehydrogenase activities were measured by the method of Wu & Racker (1959). Phosphofructokinase, triose phosphate isomerase and pyruvate kinase were assayed by the method of Bücher & Pfeleiderer (1955). Both the forward and backward reactions of 3-phosphoglycerate kinase were assayed as described by Bücher (1955). Lactate dehydrogenase was assayed by the method described by Bergmeyer (1974b). All measurements were made at 25°C.

Glycerokinase activity was assayed by the method of Bublit & Wieland (1962) at pH 9.8 and 25°C or by the combined method of Bublit & Kennedy (1954) and Bergmeyer (1963) at pH 7.4 and 37°C. The former method was also used for assay of glycerol 3-phosphate dehydrogenase with glycerol 3-phosphate as substrate instead of glycerol. Phosphatase activity was measured at pH 5.6 with 0.05M-sodium acetate buffer and at pH 9.5 with 0.02M-ethanolamine/HCl buffer, in the presence of 5 mM- MgCl_2 at 37°C, with glycerol 3-phosphate as substrate. The P_i liberated was estimated by the method of Lohmann & Jendrassik (1926).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. To ensure that all the protein was dissolved, samples were heated at 100°C for 10 min in 1M-NaOH before addition of the carbonate/copper solution, which contained no NaOH.

Chemicals and enzymes

α -Chlorohydrin was obtained from: E. Merck, Darmstadt, West Germany; Eastman/Kodak, Rochester, NY 14650, U.S.A.; The Upjohn Co., Kalamazoo, MI, U.S.A. Glycidol (2,3-epoxypropan-1-ol) was obtained from Fluka A.G., Buchs CH-9470, Switzerland, and distilled just before use. All substrates, enzymes and coenzymes used were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or Boehringer, Mannheim, West Germany. All other chemicals were of analytical grade and all solutions were made up in glass-distilled water.

Statistical analysis

Where necessary data were subjected to analysis of variance with isolation of sums of squares attribut-

able to differences between treatments and differences between ejaculated spermatozoa. The treatment-ejaculate interaction mean square has been used as the error term.

Results and Discussion

Inhibition of the oxidation of ram spermatozoan substrates in vitro

The effect was studied of α -chlorohydrin on washed ram spermatozoa metabolizing various substrates entering glycolysis and the tricarboxylate cycle at different points. A preliminary account of this aspect has been given by White *et al.* (1976).

Fructose. Fructose, which is present in high concentration in seminal plasma of the ram and is therefore a primary fuel for spermatozoal metabolism (Mann, 1964), enters the glycolytic pathway via the hexokinase reaction.

Even a concentration of α -chlorohydrin as low as 0.1 mM decreased O_2 consumption of the spermatozoa by 36% (Table 1, Fig. 1a) with fructose (5 mM) as substrate. As the concentration of α -chlorohydrin increased, the O_2 consumption decreased even further, until at 100 mM- α -chlorohydrin it was only 19% that of the control. Fructose utilization was also inhibited at 0.1 mM, but to a greater extent than O_2 consumption. This suggests that either fructose transport was affected by the α -chlorohydrin, or that the glycolytic pathway was inhibited. This is further supported by the decreasing amounts of radioactivity found in the respiratory CO_2 .

The total absence of accumulation of pyruvate and

lactate indicates that although their production was not initially inhibited completely, later during incubation, when this did occur, the accumulated lactate and pyruvate served as oxidative substrates until none remained.

An estimate of the percentage contribution made by fructose to the respiratory fuel of the spermatozoa can be obtained by calculating the dilution of the specific radioactivity of the ^{14}C derived from $[U-^{14}C]$ fructose recovered in the CO_2 . This value was substantially decreased as the concentration of α -chlorohydrin increased.

Glycerol, glycerol 3-phosphate and dihydroxyacetone. Glycerol, glycerol 3-phosphate and dihydroxyacetone enter the glycolytic sequence at the level of the triose phosphates, thereby by-passing the enzymes hexokinase, phosphofructokinase and aldolase.

When the spermatozoa metabolized glycerol (5 mM), a much higher α -chlorohydrin concentration (10 mM) was required to cause a significant decrease in O_2 consumption (Table 2, Fig. 1b); glycerol utilization, CO_2 and $^{14}CO_2$ production were also decreased at this concentration. Lactate production was decreased at a lower α -chlorohydrin concentration (1 mM) than for the other metabolic parameters measured, suggesting its continued oxidation, even though less was formed owing to inhibition of the glycolytic pathway. The relatively high concentration of α -chlorohydrin required to inhibit the metabolism of glycerol was completely unexpected, since the oxidations of glycerol 3-phosphate (Fig. 1c), dihydroxyacetone (Fig. 1d) and dihydroxyacetone phosphate were considerably inhibited by the lower

Table 1. *Effect of increasing concentrations of α -chlorohydrin on the metabolism of ram spermatozoa in vitro with fructose as substrate*

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μ Ci of $[U-^{14}C]$ -fructose (5 mM). Mean values $\pm$ S.E.M. are given for three observations. Percentage inhibition is shown in parentheses. *** $P < 0.001$ compared with control.

Concn. of α -chlorohydrin in incubation medium (mM) ...	0	0.1	1.0	10	100
Metabolic parameter (as μ mol/3 h per 10^8 spermatozoa)					
O_2 consumption	2.67 ± 0.19	$1.71 \pm 0.23^{***}$ (36%)	$0.83 \pm 0.11^{***}$ (69%)	$0.66 \pm 0.12^{***}$ (75%)	$0.51 \pm 0.02^{***}$ (81%)
CO_2 production	2.5 ± 0.3	$1.4 \pm 0.3^{***}$ (44%)	$0.8 \pm 0.2^{***}$ (68%)	$0.6 \pm 0.2^{***}$ (76%)	$0.7 \pm 0.1^{***}$ (72%)
Fructose utilized	1.65 ± 0.25	$0.44 \pm 0.09^{***}$ (73%)	$0.36 \pm 0.15^{***}$ (78%)	$0.26 \pm 0.11^{***}$ (84%)	$0.15 \pm 0.06^{***}$ (91%)
Lactate accumulated	2.11 ± 0.17	0	0	0	0
Pyruvate accumulated	0.03 ± 0.01	0	0	0	0
$10^{-2} \times ^{14}CO_2$ production (d.p.m./3 h per 10^8 spermatozoa)	740 ± 91	$401 \pm 47^{***}$ (46%)	$105 \pm 21^{***}$ (86%)	$37 \pm 24^{***}$ (95%)	$14 \pm 6^{***}$ (98%)
Specific radioactivity of $^{14}CO_2$ / Specific radioactivity of $[U-^{14}C]$ fructose $\times 100$	70	69	30	14	5

Fig. 1. Effect of increasing concentrations of α-chlorohydrin on the O₂ uptake of ram spermatozoa with various substrates (a) Fructose (5 mM), (b) glycerol (5 mM), (c) glycerol 3-phosphate (10 mM), (d) dihydroxyacetone (10 mM), (e) pyruvate (10 mM), (f) no substrate, (g) acetate (10 mM), (h) palmitate (2 mM). Washed spermatozoa were incubated for 3 h at 37°C in Warburg flasks in the presence of the following concentrations of α-chlorohydrin: control (none) (●), 0.01 mM (□), 0.1 mM (○), 1 mM (▲), 10 mM (△), 100 mM (■), no substrate or α-chlorohydrin (▼).

Table 2. Effect of increasing concentrations of α-chlorohydrin on the metabolism of ram spermatozoa in vitro with glycerol as substrate

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μCi of [U-¹⁴C]-glycerol (5 mM). Mean values ± S.E.M. are given for three observations. Percentage change is shown in parentheses.
*P < 0.05; **P < 0.01; ***P < 0.001.

Concn. of α-chlorohydrin in incubation medium (mM)	...	0	0.1	1.0	10	100
Metabolic parameter (as μmol/3 h per 10 ⁸ spermatozoa)						
O ₂ uptake		3.77 ± 0.62	3.69 ± 0.58 (2%)	3.77 ± 0.63 (0%)	1.43 ± 0.23*** (62%)	0.57 ± 0.06*** (85%)
CO ₂ production		3.0 ± 0.4	2.7 ± 0.5 (10%)	3.0 ± 0.5 (0%)	1.5 ± 0.3*** (50%)	0.7 ± 0.2*** (77%)
Glycerol utilized		1.06 ± 0.47	1.35 ± 0.36 (27%)	1.19 ± 0.76 (12%)	0.23 ± 0.20* (78%)	0.07 ± 0.06** (97%)
Lactate accumulated		0.68 ± 0.03	0.58 ± 0.11 (15%)	0.27 ± 0.06*** (60%)	0.01 ± 0.01*** (99%)	0***
Pyruvate accumulated		0.01	0.01	0.01	0	0
10 ⁻² × ¹⁴ CO ₂ production (d.p.m./3 h per 10 ⁸ spermatozoa)		744 ± 177	753 ± 176 (1%)	844 ± 186 (13%)	224 ± 47*** (70%)	54 ± 16*** (93%)
Specific radioactivity of ¹⁴ CO ₂ / Specific radioactivity of [U- ¹⁴ C]glycerol × 100		58	63	66	37	20

concentration of α -chlorohydrin, which also inhibited the metabolism of fructose. However, α -chlorohydrin and glycerol are very similar in structure, and glycerol might compete with α -chlorohydrin at the site of enzyme inhibition in the glycolytic cycle and thus offer some degree of protection. That this was the case was evident from experiments in which it was found that as the glycerol concentration was increased from 5 to 100 mM, the percentage inhibition of the O_2 uptake by the spermatozoa at 10 mM- α -chlorohydrin decreased, and at the highest glycerol concentration O_2 uptake was even slightly stimulated. The inhibition of lactate production caused by α -chlorohydrin was also decreased by increasing glycerol concentrations.

Another notable point is that of all the substrates investigated in the absence of α -chlorohydrin, glycerol stimulated the greatest O_2 uptake by ram spermatozoa, especially since glycerol utilization and lactate accumulation were relatively small. This almost certainly is due to the oxidation of some of the glycerol 3-phosphate formed into dihydroxyacetone phosphate in the mitochondria, i.e. the glycerol phosphate shuttle.

Lactate and pyruvate. Lactate, which normally accumulates even under aerobic conditions when ram spermatozoa metabolize fructose, can be converted into pyruvate, and this enters the tricarboxylate cycle directly through action of pyruvate dehydrogenase.

With lactate as substrate, there was no significant change in the gas exchange (O_2 consumption or CO_2 production) of ram spermatozoa until a concentration of 100 mM- α -chlorohydrin was reached (Table 3); a similar pattern of inhibition was obtained when accumulation of $^{14}CO_2$ and lactate utilization were

measured. Pyruvate accumulation increased at 10 mM- and 100 mM- α -chlorohydrin, perhaps indicating some inhibition of entry of pyruvate into the tricarboxylate cycle.

The O_2 consumption of ram spermatozoa with pyruvate as substrate was more sensitive to α -chlorohydrin than when lactate was used (Fig. 1e and Table 4). This difference in sensitivity was mirrored in the CO_2 and $^{14}CO_2$ production and the amount of each substrate utilized. With pyruvate as substrate, lactate accumulation by ram spermatozoa was also decreased at 1 mM- α -chlorohydrin, demonstrating that even though the utilization of pyruvate is decreased, it is not being channeled back to lactate. The greater sensitivity of the metabolism of ram spermatozoa to α -chlorohydrin with pyruvate rather than lactate as substrate is surprising, since both enter the tricarboxylate cycle through the action of pyruvate dehydrogenase.

Only at high concentrations (i.e. beyond those expected *in vivo* on administration of the drug) does α -chlorohydrin have a deleterious effect on the oxidation of pyruvate and lactate by ram spermatozoa and therefore the metabolism of these substrates is less sensitive than fructose.

Acetate, palmitate and endogenous substrate. The oxidation of the two fatty acids acetate and palmitate by ram spermatozoa was investigated to determine whether substrates that enter at the level of the tricarboxylate cycle are sensitive to α -chlorohydrin.

With acetate as substrate, the O_2 consumption of ram spermatozoa was decreased at 1 mM- α -chlorohydrin (Table 5 and Fig. 1g), whereas CO_2 and $^{14}CO_2$ production was decreased at 10 mM, indicating that the formation of acetyl-CoA from acetate is more

Table 3. Effect of increasing concentrations of α -chlorohydrin on the metabolism of ram spermatozoa *in vitro* with lactate as substrate

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μ Ci of [U- ^{14}C]-lactate (5 mM). Mean values $\pm$ S.E.M. are given for three observations ($\dagger$ two observations). Percentage change is shown in parentheses. * $P < 0.05$; ** $P < 0.01$.

Concn. of α -chlorohydrin in incubation medium (mM)	...	0	0.1	1.0	10	100
Metabolic parameter (as μ mol/3 h per 10^8 spermatozoa)						
O_2 uptake		2.41 ± 0.66	2.69 ± 0.75 (12%)	2.87 ± 0.81 (19%)	2.15 ± 0.51 (11%)	$0.84 \pm 0.14^{**}$ (65%)
CO_2 production		2.6 ± 0.9	3.2 ± 0.7 (23%)	3.2 ± 0.9 (23%)	2.4 ± 0.7 (8%)	$1.2 \pm 0.4^{**}$ (54%)
Lactate utilized		1.03 ± 0.25	1.04 ± 0.44 (1%)	1.00 ± 0.33 (3%)	0.92 ± 0.31 (11%)	$0.63 \pm 0.34^*$ (39%)
Pyruvate accumulated		0.02 ± 0.01	0.02 ± 0.01	0.06 ± 0.01	$0.18 \pm 0.06^{**}$	$0.19 \pm 0.05^{**}$
$10^{-2} \times ^{14}CO_2$ production (d.p.m./3 h per 10^8 spermatozoa) $\dagger$		519 ± 241	543 ± 260 (5%)	630 ± 328 (21%)	490 ± 241 (6%)	$85 \pm 56^{**}$ (84%)
Specific radioactivity of $^{14}CO_2$ / Specific radioactivity of [U- ^{14}C]lactate $\times 100$		76	57	65	63	32

Table 4. *Effect of increasing concentrations of α -chlorohydrin on the metabolism of ram spermatozoa in vitro with pyruvate as substrate*

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μ Ci of [U-¹⁴C]-pyruvate (10mM). Mean values $\pm$ S.E.M. are given for three observations. Percentage change is shown in parentheses. * P < 0.05; ** P < 0.01; *** P < 0.001.

Concn. of α -chlorohydrin in incubation medium (mM) ...	0	0.1	1.0	10	100
Metabolic parameter (as μ mol/3 h per 10 ⁸ spermatozoa)					
O ₂ uptake	3.26 $\pm$ 0.37	3.31 $\pm$ 0.45 (2%)	3.19 $\pm$ 0.35 (2%)	2.20 $\pm$ 0.18*** (33%)	1.88 $\pm$ 0.23*** (43%)
CO ₂ production	5.2 $\pm$ 1.0	4.9 $\pm$ 1.1 (6%)	4.3 $\pm$ 0.9 (18%)	2.4 $\pm$ 0.5* (54%)	2.3 $\pm$ 0.8** (56%)
Pyruvate utilized	5.68 $\pm$ 0.84	4.97 $\pm$ 0.68 (12%)	3.52 $\pm$ 0.61** (38%)	1.88 $\pm$ 0.39*** (67%)	1.14 $\pm$ 0.28*** (80%)
Lactate accumulated	3.41 $\pm$ 0.60	3.04 $\pm$ 0.10 (11%)	2.35 $\pm$ 0.40* (31%)	1.16 $\pm$ 0.20*** (66%)	0.99 $\pm$ 0.10*** (71%)
10 ⁻² $\times$ ¹⁴ CO ₂ production (d.p.m./3 h per 10 ⁸ spermatozoa)	520 $\pm$ 71	511 $\pm$ 78 (2%)	449 $\pm$ 54 (14%)	249 $\pm$ 22*** (52%)	186 $\pm$ 20*** (64%)

Table 5. *Effect of increasing concentrations of α -chlorohydrin on the metabolism of ram spermatozoa in vitro with acetate as substrate*

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μ Ci of [U-¹⁴C]-acetate (10mM). Mean values $\pm$ S.E.M. are given for three observations. Percentage change is shown in parentheses. ** P < 0.01; *** P < 0.001. To determine the specific radioactivity, the value of starting substrate was assumed to be 30 μ mol/flask.

Concn. of α -chlorohydrin in incubation medium (mM) ...	0	0.1	1.0	10	100
Metabolic parameter (as μ mol/3 h per 10 ⁸ spermatozoa)					
O ₂ uptake	3.03 $\pm$ 0.40	3.02 $\pm$ 0.41 (0%)	2.50 $\pm$ 0.41** (18%)	1.50 $\pm$ 0.21*** (52%)	1.41 $\pm$ 0.25*** (54%)
CO ₂ production	2.8 $\pm$ 0.6	2.9 $\pm$ 0.5 (3%)	2.6 $\pm$ 0.2 (7%)	1.2 $\pm$ 0.3*** (57%)	1.2 $\pm$ 0.2*** (57%)
Lactate accumulated	0	0	0	0	0
10 ⁻² $\times$ ¹⁴ CO ₂ production (d.p.m./3 h per 10 ⁸ spermatozoa)	391 $\pm$ 47	365 $\pm$ 61 (7%)	334 $\pm$ 44 (15%)	161 $\pm$ 30*** (59%)	123 $\pm$ 17*** (69%)
Specific radioactivity of ¹⁴ CO ₂ / Specific radioactivity of [U- ¹⁴ C]acetate $\times$ 100	86	78	75	91	62

sensitive to α -chlorohydrin than its formation from pyruvate. With palmitate as substrate (Table 6 and Fig. 1h), the O₂ consumption of ram spermatozoa was not decreased by α -chlorohydrin to the same extent as when acetate was used, but CO₂ and ¹⁴CO₂ production was more sensitive. The ¹⁴CO₂ labelling is low for the control sample compared with other substrates. This could indicate that palmitate is stimulating oxidation of endogenous lipid or that a large percentage of the palmitate is entering the general 'lipid pool' and not being oxidized directly. Acetate is a better substrate than palmitate for promoting the O₂ consumption of ram spermatozoa (see Tables 5 and 6).

When the spermatozoa metabolized endogenous substrates (probably fatty acids liberated from endogenous lipid in the absence of added substances) O₂ consumption was decreased to the value prevailing when acetate was added as substrate in the presence of α -chlorohydrin.

The fact that the enzyme system responsible for converting pyruvate into acetyl-CoA is less sensitive to α -chlorohydrin than the one converting fatty acids into acetyl-CoA is borne out also by the finding that inhibition of oxidation of endogenous substrate is similar to that of acetate. Thus it appears that there is a second, less potent site which is sensitive to α -chlorohydrin, i.e. where fatty acids are converted into

Table 6. *Effect of increasing concentrations of α -chlorohydrin on the metabolism of ram spermatozoa in vitro with palmitate as substrate*

Washed spermatozoa were incubated aerobically for 3 h at 37°C in Warburg flasks in the presence of 0.5 μ Ci of [U-¹⁴C]-palmitate (2mM). Mean values $\pm$ S.E.M. are given for three observations. Percentage change is shown in parentheses. ***P* < 0.01; ****P* < 0.001. To determine the specific radioactivity, the value of starting substrate was assumed to be 6 μ mol/flask.

Concn. of α -chlorohydrin in incubation medium (mM)	...	0	0.1	1.0	10	100
Metabolic parameter (as μ mol/3 h per 10 ⁸ spermatozoa)						
O ₂ uptake		1.89 $\pm$ 0.06	1.88 $\pm$ 0.08 (0%)	1.74 $\pm$ 0.08 (8%)	1.26 $\pm$ 0.18*** (33%)	1.36 $\pm$ 0.18*** (28%)
CO ₂ production		2.1 $\pm$ 0.4	2.4 $\pm$ 0.5 (+14%)	1.9 $\pm$ 0.3 (10%)	1.2 $\pm$ 0.3** (43%)	1.4 $\pm$ 0.3** (33%)
Lactate accumulated		0	0	0	0	0
¹⁴ CO ₂ production (d.p.m./3 h per 10 ⁸ spermatozoa)		3603 $\pm$ 4	3624 $\pm$ 9 (0%)	2619 $\pm$ 22*** (27%)	988 $\pm$ 17*** (73%)	705 $\pm$ 105*** (80%)
Specific radioactivity of ¹⁴ CO ₂ Specific radioactivity of [U- ¹⁴ C]palmitate $\times$ 100	17		15	14	8	5

acetyl-CoA to enter the tricarboxylate cycle. The primary site appears to be between glycerol 3-phosphate and pyruvate in the glycolytic pathway.

Location of the site of disruption of the glycolysis of ram spermatozoa by α -chlorohydrin in vitro

From the previous experiments, it appeared that the site of action of α -chlorohydrin was on the glycolytic pathway rather than the subsequent tricarboxylate cycle.

The present experiments were designed to establish the specific site of the inhibition by measuring the concentrations of glycolytic intermediates and the activities of glycolytic enzymes in ejaculated ram spermatozoa *in vitro*. Preliminary accounts of this work have been given (Suter *et al.*, 1975; Mohri *et al.*, 1975).

Effect of α -chlorohydrin on concentrations of glycolytic intermediates. Washed ram spermatozoa, supplemented with 15mM-fructose, were incubated for 3h in the presence and absence of 3mM- α -chlorohydrin and the concentrations of glycolytic intermediates and adenosine nucleotide phosphates were measured subsequently. The results (Table 7) show that α -chlorohydrin caused accumulation of fructose 1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, and disappearance of pyruvate and lactate. Glucose 6-phosphate and fructose 6-phosphate were also somewhat decreased. The amounts of 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate were too small to be determined in both the control and the experimental spermatozoa. Phosphoglycerate formation from glyceraldehyde 3-phosphate is obviously the site of action of α -chlorohydrin and this step in-

Table 7. *Glycolytic and related intermediates in ram spermatozoa after incubation in the presence and absence of α -chlorohydrin*

Washed spermatozoa were incubated for 3 h at 37°C in Warburg flasks with fructose (15mM) as substrate. N.D., Not detectable. Values (nmol/10⁹ spermatozoa) are the means $\pm$ S.E.M. of five determinations.

Intermediate	α -Chlorohydrin (mM)	
	0	3
Glucose 6-phosphate	33.7 $\pm$ 4.9	17.4 $\pm$ 1.8
Fructose 6-phosphate	12.8 $\pm$ 1.5	6.6 $\pm$ 0.9
Fructose 1,6-bisphosphate	1.5 $\pm$ 0.2	49.2 $\pm$ 0.4
Dihydroxyacetone phosphate	9.2 $\pm$ 1.1	26.6 $\pm$ 1.5
Glyceraldehyde 3-phosphate	0.5 $\pm$ 0.1	1.6 $\pm$ 0.5
3-Phosphoglycerate	N.D.	N.D.
2-Phosphoglycerate	N.D.	N.D.
Phosphoenolpyruvate	N.D.	N.D.
Pyruvate	41.2 $\pm$ 0.6	2.1 $\pm$ 0.1
Lactate	17440 $\pm$ 1180	64 $\pm$ 53
ATP	177 $\pm$ 14	18.7 $\pm$ 3.1
ADP	5.8 $\pm$ 2.3	5.4 $\pm$ 4.9
AMP	14.4 $\pm$ 8.6	203 $\pm$ 8.0

volves two enzymes, i.e. glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase. The intermediate compound, 1,3-bis-phosphoglycerate, produced by the action of the former enzyme is extremely unstable and rapidly breaks down to 3-phosphoglycerate. However, there was no accumulation of this intermediate in the presence of α -chlorohydrin, so that it is more likely that glyceraldehyde 3-phosphate dehydrogenase rather than 3-phosphoglycerate kinase is the enzyme inhibited by the drug. There was no difference between the results obtained

with non-distilled or twice-distilled samples of α -chlorohydrin.

Inhibition of glycolysis at this point would prevent production of ATP required for spermatozoa motility by this pathway. The results show that there was indeed an almost total loss of ATP after α -chlorohydrin treatment, it being quantitatively converted into AMP by adenylate kinase.

Direct effect of α -chlorohydrin on glycolytic enzymes. An attempt was made to see if α -chlorohydrin inhibits glyceraldehyde 3-phosphate dehydrogenase in a cell-free system, by using sonicated spermatozoal preparations as the enzyme source (see the Materials and Methods section). However, α -chlorohydrin included in the reaction cuvette had practically no effect on the activity of this enzyme or any of the other glycolytic enzymes, including hexokinase, phosphofructokinase, aldolase, triosephosphate isomerase, 3-phosphoglycerate kinase (both forward and backward reaction), pyruvate kinase and lactate dehydrogenase at concentrations as high as 300 mM (Table 8). With glyceraldehyde 3-phosphate dehydrogenase, the assay method used in the present experiment is said to be unsatisfactory in crude preparations because of the ubiquity of enzymes which may involve substrates, coenzymes and products in other reactions (Krebs, 1955). The experiment was therefore repeated with the crystalline enzyme from rabbit muscle purchased from Sigma Chemical Co., but the results were also negative.

Although twice-distilled α -chlorohydrin decreased the activity of the crystalline enzyme by 25% at 300 mM, non-distilled α -chlorohydrin inhibited activity by 65% at 30 mM. It is suggested from these results that some unknown compound(s), probably derived from α -chlorohydrin and possibly a true inhibitor, was present as an impurity in the non-distilled α -chlorohydrin. In another experiment, sonicated preparations from spermatozoa suspended in 50 mM-Tris/HCl buffer, pH 7.4, were preincubated with different concentrations of α -chlorohydrin at 37°C

for 1 h, and then assayed for glyceraldehyde 3-phosphate dehydrogenase activity. After preincubation with 100 mM- α -chlorohydrin (twice-distilled), enzyme activity of the spermatozoal preparation was inhibited to some extent, but the same concentration of α -chlorohydrin added to the preparation in the cuvette had no effect. These results suggest that α -chlorohydrin does not inhibit glyceraldehyde 3-phosphate dehydrogenase by direct action on the enzyme. It is possible, however, that a metabolic product of α -chlorohydrin might be responsible for the inhibition of the enzyme as indicated by the 'cross-over' point obtained by the measurements of glycolytic intermediates.

Effect of α -chlorohydrin on activity of glycolytic enzymes. Washed ram spermatozoa were incubated under the same conditions as those used for measurements of glycolytic intermediates, and the activities of glycolytic enzymes in the spermatozoa were measured after separation from the reaction medium. The activities of triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase were decreased by 80% and 95% respectively in the presence of 3 mM- α -chlorohydrin, whereas those of other enzymes were not significantly changed even when compared with those of the enzymes before the incubation (Table 9). In this particular experiment, aldolase activity was inhibited by α -chlorohydrin and control activities of phosphofructokinase and 3-phosphoglycerate kinase were lowered after the 3 h incubation. From the results of a similar experiment in which a range of concentrations of α -chlorohydrin was used, it was obvious that glyceraldehyde 3-phosphate dehydrogenase is the most sensitive enzyme, followed by triosephosphate isomerase. Glyceraldehyde 3-phosphate dehydrogenase activity was inhibited by 60% in the presence of 0.03 mM- α -chlorohydrin and was not detectable at 30 mM. Among the other enzymes tested, phosphofructokinase was slightly affected at 30 mM- α -chlorohydrin, as were both aldolase and lactate dehydrogenase at 300 mM.

Table 8. *Direct effect of α -chlorohydrin on activity of glycolytic enzymes from ram spermatozoa*

α -Chlorohydrin was added to sonicated preparations of spermatozoa in semen in the reaction cuvette used for the enzyme assay. Values (μ mol/min per 10^9 spermatozoa at 25°C) are the means of duplicate determinations from a pool of three ram ejaculates. The differences between the duplicate determinations are within 5%.

Enzyme	α -Chlorohydrin (mM)					
	0	0.1	1	10	100	300
Hexokinase	0.59	0.59	0.59	0.60	0.59	0.57
Phosphofructokinase	0.37	0.37	0.37	0.37	0.37	0.34
Aldolase	0.42	—	0.41	0.41	0.42	0.36
Triose phosphate isomerase	3.77	—	—	3.80	3.86	3.51
Glyceraldehyde 3-phosphate dehydrogenase	0.56	—	—	0.55	0.56	0.51
3-Phosphoglycerate kinase	3.63	—	3.54	3.70	3.61	3.30
Pyruvate kinase	0.51	—	0.51	0.51	0.52	0.50
Lactate dehydrogenase	6.75	—	—	6.77	6.77	6.58

Table 9. *Activity of glycolytic enzymes in ram spermatozoa incubated in the presence and absence of α -chlorohydrin (3 mM)*
 Washed spermatozoa were incubated for 3 h at 37°C in Warburg flasks with fructose (15 mM) as substrate and the activity of glycolytic enzymes was measured in the spermatozoa after separation from the reaction medium. Values ($\mu\text{mol/min per } 10^9$ spermatozoa at 25°C) are the means $\pm$ S.E.M. of three determinations.

Enzyme	Time (h)		
	0	3	
		Control	α -Chlorohydrin
Hexokinase	0.48 $\pm$ 0.04	0.48 $\pm$ 0.02	0.51 $\pm$ 0.05
Phosphofructokinase	0.76 $\pm$ 0.11	0.23 $\pm$ 0.06	0.53 $\pm$ 0.09
Aldolase	0.36 $\pm$ 0.04	0.37 $\pm$ 0.02	0.23 $\pm$ 0.03
Triose phosphate isomerase	4.28 $\pm$ 0.34	3.45 $\pm$ 0.44	0.72 $\pm$ 0.05
Glyceraldehyde 3-phosphate dehydrogenase	0.55 $\pm$ 0.03	0.45 $\pm$ 0.01	0.03 $\pm$ 0.01
3-Phosphoglycerate kinase	3.59 $\pm$ 0.13	2.13 $\pm$ 0.24	3.07 $\pm$ 0.02
Pyruvate kinase	0.48 $\pm$ 0.04	0.52 $\pm$ 0.03	0.49 $\pm$ 0.14
Lactate dehydrogenase	8.50 $\pm$ 0.64	8.71 $\pm$ 0.21	9.02 $\pm$ 0.08

The impairment of spermatozoal motility observed at the end of incubation paralleled the decrease in the activity of glyceraldehyde 3-phosphate dehydrogenase. Thus the results agree with the data obtained from the measurements of intermediates and reveal the indirect action of α -chlorohydrin on both this enzyme and triose phosphate isomerase. Although the isomerase activity was severely decreased by 3 mM- α -chlorohydrin, the remaining activity seems sufficient for the conversion of dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, because the ratio of these intermediates shown in Table 7 is near the value at the equilibrium of this reaction, i.e. 22:1.

The above experiments were done with the spermatozoa separated from the suspending medium. It is therefore conceivable that α -chlorohydrin might act indirectly by modifying the membrane structure of spermatozoa, causing specific leakage from the spermatozoal cells of the enzymes observed to have decreased activities. Further, the separation procedure, although only short, was conducted in the cold. It is well known that 'cold shock' leads to leakage of glycolytic enzymes such as lactate dehydrogenase from mammalian spermatozoa (Murdoch & White, 1968; Harrison & White, 1972). To check this possibility, spermatozoal suspensions were dialysed against 0.25 M-sucrose containing 1 mM-2-mercaptoethanol and 1 mM-EDTA solution and then sonicated as described above after incubation with 15 mM-fructose in the presence and absence of 3 mM- α -chlorohydrin. The preparation was then used for assay of enzymes. The results did not indicate enzyme leakage, although the activity of lactate dehydrogenase was somewhat higher in dialysed suspensions than in centrifuged spermatozoa.

In another series of experiments, the incubation medium, containing different concentrations of α -chlorohydrin (twice-distilled) was separated from the spermatozoa and then examined for inhibitory

action on glyceraldehyde 3-phosphate dehydrogenase in a spermatozoal enzyme preparation separately prepared. It was hoped that one or more metabolic products of α -chlorohydrin would be detected and that these might include the true inhibitor. This was, however, not the case.

Effect of α -chlorohydrin on enzymes of glycerol metabolism. Since the chemical structure of α -chlorohydrin is similar to that of glycerol, the effect of this drug on some enzymes involved in glycerol metabolism was also examined, by using sonicated spermatozoal preparations. Glycerokinase, which phosphorylates glycerol to glycerol 3-phosphate, was inhibited by α -chlorohydrin at concentrations above 10 mM and was totally inhibited at 300 mM, when the activity was measured at pH 9.8 as described in the Experimental section. However, it was soon realized that the effect was due to inhibition of the NAD⁺-dependent glycerol 3-phosphate dehydrogenase from rabbit muscle, which was added to the assay mixture as a coupling enzyme. The activity of glycerokinase, when measured at pH 7.4 in the system containing no coupling enzyme, was stimulated by α -chlorohydrin at concentrations exceeding 10 mM. During the course of this experiment, α -chlorohydrin contained traces (0.13% in the non-distilled sample and 0.016% in twice-distilled) of glycerol, but since the K_m for glycerol is 10 μ M-100 and at least 2.5 mM-glycerol was always present in the assay mixture, this cannot explain the stimulation of glycerokinase activity at high α -chlorohydrin concentrations.

It is not certain whether glycerol 3-phosphate dehydrogenase present in ram spermatozoa is also inhibited by α -chlorohydrin. The activity of this enzyme in the cytosol, however, was very low compared with that of mitochondrial glycerol 3-phosphate oxidase (Mohri *et al.*, 1965), which is present in the spermatozoal enzyme preparation and was not affected by α -chlorohydrin.

When measured with glycerol 3-phosphate as substrate, alkaline phosphatases in spermatozoa and seminal plasma were inhibited by α-chlorohydrin above 10 mM. Acid phosphatases in spermatozoa and in seminal plasma, on the other hand, were quite resistant to α-chlorohydrin.

Effect of injected α-chlorohydrin on the glycolytic activity of spermatozoa collected from rams. The experiments in this section were designed to investigate whether α-chlorohydrin, administered to rams by injection at a dosage known to cause infertility (Brown-Woodman *et al.*, 1974), would cause the same metabolic changes in the spermatozoa *in vivo* as those found *in vitro* and therefore substantiate the suggested mode of action deduced from experiments *in vitro*.

Effect of injected α-chlorohydrin on glycolytic and related intermediates in ejaculated ram spermatozoa. Concentrations of intermediates (nmol/10⁹ spermatozoa) before, during and after intramuscular injection of six rams with α-chlorohydrin (25 mg/kg body wt., daily for 4 days) are presented in Table 10. In this experiment the concentrations of 3-phosphoglycerate were determined, and 24 h after the first injection (day 2), the inhibited step between triose phosphate and 3-phosphoglycerate was the same as that found when ram spermatozoa were treated with α-chlorohydrin *in vitro*. The decrease in fructose 1,6-bisphosphate in spermatozoa collected on days 4 and 5 compared with day 2 suggested that some hexose carbon was metabolized via glyceraldehyde 3-phosphate into the pentose phosphate pathway. At 1 month after the final injection, however, the intermediates in the spermatozoa had returned to near pre-injection concentrations and normal lactate accumulation indicated that glycolysis was no longer blocked. Spermatozoal L-glycerol 3-phosphate (Table 10) increased in parallel with fructose 1,6-bisphosphate and triose phosphate during the injection period, but remained higher than pre-injection concentrations even on day 31 of the recovery period.

Adenine nucleotide concentrations in spermatozoa were also recorded during the same period. The dramatic 92% fall in ATP 24 h after the first injection of α-chlorohydrin (day 2) was clearly devastating for the spermatozoa, but contradicts the findings of Chulavatnatol *et al.* (1977) in the rat. It also indicated that ram spermatozoa depend very much on glycolysable sugar as their normal source of energy for maintenance and production of ATP. Spermatozoal ATP concentrations remained very low on day 5 of recovery, but astonishingly were still only 38% of the pre-injection value on day 31 when glycolysis was no longer affected. This is difficult to understand, since the motility of the spermatozoa ejaculated at this time was as good as before the injection of α-chlorohydrin.

However, there is evidence that in ejaculated mon-

Table 10. Concentrations of glycolytic and related intermediates in ejaculated ram spermatozoa before, during and after injecting rams with α-chlorohydrin. Values (nmol/10⁹ spermatozoa) are the means ± s.e.m. of six determinations. Six rams were injected with α-chlorohydrin (25 mg/kg intramuscularly, daily for 4 days) and spermatozoa were collected, washed and incubated for 3 h at 37°C in Warburg flasks with fructose (15 mM) as substrate. N.D., Not detectable by standard spectrophotometric (S) techniques or a fluorimetric (F) method.

Period	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-bisphosphate	Triose phosphate	Glycerol 3-phosphate	3-Phosphoglycerate	Lactate	ATP	ADP	AMP
Pre-injection	3.9 ± 2.4	2.0 ± 0.5	4.8 ± 1.3	5.2 ± 1.3	N.D. (S)	2.3 ± 0.9	902.2 ± 117.8	68.7 ± 12.3	116.7 ± 17.6	143.8 ± 31.2
Injection (day 2)	22.3 ± 2.9	7.3 ± 1.0	174.2 ± 14.1	51.0 ± 3.1	80.8 ± 11.0	N.D. (F)	86.3 ± 21.5	5.7 ± 0.9	68.0 ± 8.5	278.7 ± 27.3
Injection (day 4)	19.7 ± 3.9	5.2 ± 1.3	103.5 ± 20.5	35.5 ± 5.7	75.3 ± 32.1	N.D. (F)	99.5 ± 42.5	4.0 ± 0.7	8.0 ± 3.0	389.3 ± 95.0
Post-injection (day 5)	20.8 ± 3.6	6.8 ± 1.3	64.2 ± 14.6	41.0 ± 6.1	53.7 ± 6.8	N.D. (F)	50.0 ± 7.5	6.5 ± 1.5	26.2 ± 4.3	183.7 ± 37.9
Post-injection (day 31)	4.0 ± 0.4	N.D. (S)	3.1 ± 0.8	3.4 ± 1.0	9.2 ± 2.9	—	845.7 ± 160.6	25.7 ± 6.8	110.3 ± 8.1	178.2 ± 23.4

key (Hoskins *et al.*, 1971), bull (Garbers *et al.*, 1973) and boar (Garbers *et al.*, 1973) spermatozoa, glycolytic rates and motility are inversely proportional to the energy charge of the spermatozoa (Atkinson, 1968). It is quite conceivable therefore that the glycolytic rate of these 'recovered' spermatozoa is much greater than before injection, because many of the spermatozoa may have been partially damaged previously by the presence of α -chlorohydrin. At this stage of recovery the spermatozoa may be using greater than normal quantities of ATP for repair processes.

During the injection period the fall in ATP and ADP concentrations in the spermatozoa was reflected in a quantitative conversion into AMP, but the decrease in total adenine nucleotides seen on day 5 suggested some net adenine nucleotide breakdown.

The low [ATP]/([ADP] + [AMP]) ratio found in the pre-injection spermatozoa was unexpected when compared with ratios found in most mammalian tissues. However, similar results have been found in ejaculated monkey and bull spermatozoa (Hoskins *et al.*, 1971; Newton & Rothschild, 1961). These findings may in part be due to the difficulty of rapidly separating spermatozoa from seminal plasma without causing undue stress to the cells. Mann (1964) has reported considerably higher ATP concentrations (220 nmol/10⁹ spermatozoa) in ram semen (spermatozoa + seminal plasma). This does not, however, have any important bearing on the effect of α -chlorohydrin on spermatozoal adenine nucleotides, except that the [ATP]/([ADP] + [AMP]) ratios determined may be less than those actually present *in vivo*.

Effect of injected α -chlorohydrin on activities of glycolytic enzymes in spermatozoa. The activities of glycolytic enzymes in sonicated preparations of spermatozoa collected from rams before, during and after injection with α -chlorohydrin are shown in Table 11. During the injection period, spermatozoal glyceraldehyde 3-phosphate dehydrogenase activity was decreased so much that it was not detectable by the method used. Spermatozoal triose phosphate isomerase and aldolase activities were also both severely decreased, but none of the other glycolytic enzymes measured were affected by injection of the drug. During the post-injection period the activities of glyceraldehyde 3-phosphate dehydrogenase and aldolase increased in the spermatozoa until day 26, when recovery was essentially complete. Spermatozoal triose phosphate isomerase activities also recovered during this period, but were still only 77% of the pre-injection values on day 26. The activity of this enzyme, however, is very large compared with the activities of other glycolytic enzymes, and it is unlikely that this extent of inhibition, on day 26, would have any significant effect on spermatozoal glycolysis. The large standard errors in enzyme activities during

Table 11. Activities of glycolytic enzymes in spermatozoa collected from rams before, during and after injection with α -chlorohydrin

Values (μ mol/min per 10⁹ spermatozoa at 25°C) are the means $\pm$ s.e.m. Five animals were involved in this experiment but ejaculates were not always available from each ram; the number of ejaculates used for each test is therefore shown in parentheses. Washed spermatozoa were incubated for 3 h at 37°C in Warburg flasks with fructose (15 mM) as substrate and the activity of glycolytic enzymes was measured in the spermatozoa after separation from the reaction medium. N.D., Not detectable.

Period	Hexokinase	Phospho-fructokinase	Aldolase	Triose phosphate isomerase	Glyceraldehyde 3-phosphate dehydrogenase	3-Phosphoglycerate kinase	Pyruvate kinase	Lactate dehydrogenase
Pre-injection (5)	0.37 $\pm$ 0.03	0.52 $\pm$ 0.08	0.41 $\pm$ 0.02	5.58 $\pm$ 0.56	0.40 $\pm$ 0.01	2.96 $\pm$ 0.42	0.66 $\pm$ 0.06	7.31 $\pm$ 0.18
Injection (day 2) (3)	0.46 $\pm$ 0.08	0.50 $\pm$ 0.08	0.25 $\pm$ 0.05	0.72 $\pm$ 0.28	N.D.	2.74 $\pm$ 0.17	0.89 $\pm$ 0.14	7.57 $\pm$ 0.79
Injection (day 4) (1)	0.35	0.44	0.13	0.45	N.D.	3.02	0.41	6.97
Post-injection (day 4) (4)	0.36 $\pm$ 0.09	0.25 $\pm$ 0.13	0.14 $\pm$ 0.07	0.20 $\pm$ 0.02	0.002 $\pm$ 0.001	2.88 $\pm$ 0.86	0.48 $\pm$ 0.08	6.41 $\pm$ 0.59
Post-injection (day 11) (4)	0.59 $\pm$ 0.03	0.52 $\pm$ 0.07	0.30 $\pm$ 0.09	2.70 $\pm$ 1.02	0.23 $\pm$ 0.09	3.84 $\pm$ 0.49	0.68 $\pm$ 0.04	7.88 $\pm$ 0.19
Post-injection (day 18) (5)	0.51 $\pm$ 0.08	0.70 $\pm$ 0.22	0.37 $\pm$ 0.07	3.45 $\pm$ 0.67	0.29 $\pm$ 0.08	4.12 $\pm$ 0.91	0.62 $\pm$ 0.07	7.27 $\pm$ 1.09
Post-injection (day 26) (3)	0.59 $\pm$ 0.12	0.51 $\pm$ 0.10	0.44 $\pm$ 0.03	4.28 $\pm$ 0.25	0.38 $\pm$ 0.02	2.63 $\pm$ 0.48	0.77 $\pm$ 0.08	6.75 $\pm$ 0.48

this period were due to differing rates of recovery of the enzyme activities in individual animals.

The motility of the ejaculated spermatozoa was monitored throughout the whole experiment and was always found to parallel the activity of glyceraldehyde 3-phosphate dehydrogenase. However, the spermatozoa never became completely immotile at any time during or after α -chlorohydrin treatment, suggesting that some energy was still available through oxidation of endogenous substrates. These are likely to be fatty acids derived from endogenous lipid and have been shown above to be relatively insensitive to α -chlorohydrin. No other glycolytic enzymes were significantly affected at any time after the injection of α -chlorohydrin.

Effect of glycidol on glycolysis and spermatozoal enzyme activities in vitro. It has been proposed that glycidol may be responsible for the anti-fertility action of α -chlorohydrin on spermatozoa within the epididymis of the rat, because of the ease with which α -chlorohydrin may be converted into glycidol and also because glycidol itself has been shown to have anti-fertility properties (Jackson *et al.*, 1970). The effects of this compound on the metabolism of ram spermatozoa have therefore been investigated with this in mind. A very preliminary account of the latter work has been given by Suter & Mohri (1975).

Effect of glycidol on [U- 14 C]fructose (7.5 mM) oxidation. The complete oxidation of [U- 14 C]fructose to CO_2 by ram spermatozoa, as determined by the recovery of radioactivity in respiratory CO_2 , was unaltered by glycidol (0.1 mM or 1.0 mM), but inhibited by 45% and 67% at 10 mM- and 100 mM-glycidol respectively. This is in contrast with the inhibition of [U- 14 C]fructose oxidation by α -chlorohydrin, which was already considerable at 0.1 mM. The O_2 uptake of the spermatozoa, however, was not significantly decreased even at 100 mM-glycidol. Fructose utilization was extremely variable in the sample tested, although a decrease is suggested in the presence of glycidol. Lactate production by the spermatozoa tended to be inhibited by 1.0 mM- and 10.0 mM-glycidol, but not by 100 mM-glycidol.

The decreased complete oxidation of fructose to CO_2 , whereas the respiratory rate remained unaltered, coupled with no decrease in lactate production at 100 mM-glycidol, suggests the presence of an alternative substrate competing with fructose. Glycidol is known to be hydrolysed slowly to glycerol on standing, and care was taken always to use freshly distilled glycidol in these experiments. For example, flasks incubated with 100 mM-glycidol contained only 0.046 μmol of glycerol before incubation. However, this does not remove the possibility of increased glycerol formation during incubation, and experiments showed that this was indeed the case. The concentration of glycerol in flasks containing 100 mM-glycidol was 2.95 mM after 3 h incubation. Although

we have no data on competitive studies between oxidation of glycerol and fructose by ram spermatozoa, they do have a considerably higher respiratory rate when offered glycerol at a saturating concentration (Figs. 1a, 1b). At glycidol concentrations of 10 and 100 mM, the dilution of [U- 14 C]fructose radioactivity found in the respiratory CO_2 was almost certainly due to oxidation of non-radioactively labelled glycerol via pyruvate and the tricarboxylate cycle. The excess of pyruvate formed, for example at 100 mM-glycidol, would be rapidly converted into lactate.

Glycidol does inhibit glycolysis, but the inhibition site probably lies between the conversion of fructose into triose phosphate, and glycidol therefore does not appear to be an active metabolite of α -chlorohydrin. This point was then further investigated by observing the effect of glycidol on some glycolytic enzymes in ram spermatozoal sonicated preparations, including those inhibited by a metabolite of α -chlorohydrin.

Effect of glycidol on enzyme activities. Glycidol (100 mM) had no significant effect on any of the enzymes tested when added directly to spermatozoal enzyme preparations. Its failure to inhibit aldolase, triose phosphate isomerase or glyceraldehyde 3-phosphate dehydrogenase directly shows that it is not an active metabolite of α -chlorohydrin in ram spermatozoa. In addition its inhibitory effect on the glycolytic and respiratory activity of these spermatozoa was not associated with any direct action on hexokinase, lactate dehydrogenase or glycerokinase.

General Discussion and Conclusions

Site of α -chlorohydrin inhibition on spermatozoal metabolism and its mechanism of anti-fertility action

Administration of high doses of α -chlorohydrin to the rat causes lesions in the caput epididymis and irreversible blockage of the passage of spermatozoa from the testis to the epididymis (Ericsson & Connor, 1969; Brown-Woodman *et al.*, 1976). However, the precise mechanism by which a non-lesion-forming dose of α -chlorohydrin exerts its temporary anti-fertility effect in male animals has hitherto been obscure and the evidence sometimes conflicting. Thus it was reported that the drug has no effect on spermatozoal motility when given orally to rats (Ericsson, 1969). Other investigators have observed decreased spermatozoal motility, and suggested either disturbance in phospholipid synthesis or metabolic failure of the spermatozoa due to an effect of α -chlorohydrin in the male reproductive tract (Coppola, 1969; Samojlik & Chang, 1970). Evidence has been accumulating to indicate that the effects of the drug are at least initially on spermatozoa in the epididymis (Crabo & Appellgren, 1972; Brown & White, 1973; Brown-Woodman & White, 1974, 1975), resulting in inhibition of spermatozoa transport in

the female genital tract by virtue of a decrease in both spermatozoal motility and possibly uterine contractility (Vickery *et al.*, 1974).

Our previous work has revealed that administration of α -chlorohydrin causes disruption of fructose and glucose metabolism not only in epididymal spermatozoa of the rat but also in ejaculated spermatozoa of the ram (Brown & White, 1973; Brown-Woodman & White, 1974, 1975, 1976a). In the latter case the oxidation of glucose by spermatozoa collected from rams injected with α -chlorohydrin was significantly suppressed within 1–4 days, but gradually returned to normal 3 or 4 weeks after the last injection (Brown & White, 1973; Brown-Woodman & White, 1976a). Further, the decline and restoration of the metabolic activity of the spermatozoa corresponded to the loss and subsequent return of fertility.

Our initial studies had also shown that α -chlorohydrin at relatively low (0.1 mM) concentrations exerted a similar inhibitory effect on the oxidation of fructose when incubated with normal ejaculated ram spermatozoa *in vitro* (Brown-Woodman *et al.*, 1975; Brown-Woodman & White, 1976b) and almost completely blocked aerobic glycolysis *in vitro*. The inhibition of spermatozoal glycolysis was similar to that obtained *in vivo* (Brown & White, 1973; Brown-Woodman & White, 1976a) when rams were injected daily with 25 mg of α -chlorohydrin/kg and the metabolism of the ejaculated spermatozoa was studied. As the O_2 consumption of the spermatozoa was decreased 24 h after the first injection, the site of action must be the epididymis, in which spermatozoa are stored before ejaculation. If one assumes even distribution of this drug over 80% of the body, then the concentration of α -chlorohydrin to which the spermatozoa would be exposed in the epididymis would be $25/110 \times 1000/800 = 0.182$ mM, where 110 = mol.wt. of α -chlorohydrin, 25 = dosage of α -chlorohydrin (mg/kg body wt.) and it is assumed that 80% of the body weight is fluid. However, concentrations *in vivo* could be expected to be higher, since α -chlorohydrin has been shown to be concentrated in the cauda epididymis (Crabo & Appelgren, 1972; Edwards *et al.*, 1975).

Although in the present experiment the oxidation of glycerol 3-phosphate, dihydroxyacetone and dihydroxyacetone phosphate by ram spermatozoa was also decreased by α -chlorohydrin (0.1 mM) *in vitro*, the oxidation of pyruvate, lactate and endogenous substrates was little affected at these concentrations. These experiments show that the metabolism of fructose by ram spermatozoa is much more susceptible to α -chlorohydrin than that of substrates lower down the glycolytic pathway or those entering the tricarboxylate cycle. Such data suggest that the primary site of action of α -chlorohydrin is in the glycolytic pathway, although higher concentrations

of α -chlorohydrin may affect other reactions, including those involved in the tricarboxylate cycle, perhaps by non-specific alkylation (Jones *et al.*, 1969). Both the studies on the effect of α -chlorohydrin on ram spermatozoa *in vitro* and the studies on spermatozoa of rams injected with α -chlorohydrin show that glycolysis is blocked between production of triose phosphate and phosphoglycerate formation.

The experiments further establish that the specific site of the inhibition is the enzyme glyceraldehyde 3-phosphate dehydrogenase and to a lesser extent triose phosphate isomerase and aldolase. It is suggested that the spermatozoa are rendered infertile because their most important source of energy for ATP synthesis, namely oxidation of glycolysable sugar, is no longer possible. ATP is therefore not available for motility of the spermatozoa at two essential stages, i.e. during passage through the cervix and for fertilization. In addition ATP is not available for the maintenance of other normal energy-requiring processes that are essential for cell viability.

Our conclusion does not conflict with the report that the spermatozoa of α -chlorohydrin-treated animals, recovered from the uterus of mated females and then transferred directly into the ovarian bursa, were capable of fertilization (Vickery *et al.*, 1974), because endogenous respiration, probably arising from lipid oxidation, was not so susceptible to the drug. The motility of spermatozoa obtained from rams injected with α -chlorohydrin, although severely affected, was not, however, completely abolished (Brown-Woodman *et al.*, 1974), and this residual motility may be sufficient for the penetration of a spermatozoon into an ovum, provided that there is no acrosomal damage.

The question arises as to how α -chlorohydrin causes the observed decrease in the activities of glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase. When added directly to the reaction cuvettes containing the sonicated spermatozoal preparation, the drug did not affect these enzymes or other glycolytic enzymes at the low concentrations found to be so effective in decreasing fructose oxidation by intact spermatozoa. The activities of these enzymes were, however, considerably suppressed after the incubation of spermatozoa with α -chlorohydrin at such concentrations and caused the accumulation of glycolytic intermediates before conversion of fructose 1,6-bisphosphate and triose phosphate into phosphoglycerate. This implies that the effect is not a direct one.

It seems most likely that glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase may be inhibited competitively by a metabolic product of α -chlorohydrin, which fits the active site by virtue of its structural similarity to the true substrates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Glycidol, which has been shown to have

a similar anti-fertility effect to α -chlorohydrin (Jackson *et al.*, 1970), had no direct action on these enzymes (Suter *et al.*, 1975) and is clearly not the metabolic product concerned. The metabolic and anti-fertility effects of glycidol remain obscure, but glycidol may share with α -chlorohydrin the necessity to form an active intermediate that is produced only after contact of the compound with intact spermatozoa.

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