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# In vitro inhibition of rat cauda epididymal sperm glycolytic enzymes by ornidazole, α-chlorohydrin and 1-chloro-3-hydroxypropanone

W. BONE and T. G. COOPER

Institute of Reproductive Medicine of the University, Domagkstrasse 11, D-48129 Münster, Germany

## Summary

Chlorinated antifertility compounds are known to inhibit glycolysis of spermatozoa as they reside in the epididymis but new compounds need to be evaluated that retain antifertility action but do not exhibit side-effects. In this study, two known antifertility agents and a related compound were compared for their inhibition of rat sperm metabolism and motility in vitro. The dose-dependent inhibition in vitro of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triose-phosphate isomerase (TPI) of distal cauda epididymal rat spermatozoa by (*R*)-, (*S*)- and (*R*,*S*)-ornidazole (ORN), (*R*,*S*)-α-chlorohydrin (ACH) and 1-chloro-3-hydroxy-propanone (CHOP) was compared. The direct inhibition of GAPDH by ORN suggests that it inhibits without prior conversion outside the cell but inhibition was not stereo-specific. The GAPDH, but not TPI, activity of spermatozoa incubated with ACH and CHOP was highly correlated with kinematic parameters of spermatozoa incubated in pyruvate- and lactate-free medium. ACH only inhibited the activity of intact spermatozoa and the inhibition was not reversed by washing the particulate sperm fraction after sonication. High concentrations of ACH (100 mmol/L) killed intact rat spermatozoa and decreased the extent of GAPDH inhibition. CHOP, unlike ACH, was an effective inhibitor of both intact and sonicated cells. Pre-CHOP, the dimethylketal precursor of CHOP, and its other hydrolysis product MeOH, were both ineffective in vitro. CHOP and related ketals may be more effective inhibitors of sperm glycolysis than ACH and may prove useful for investigating sperm-specific glycolytic inhibition, a prerequisite for the development of antiglycolytic, post-testicular acting contraceptives.

**Keywords:** chlorinated antifertility agents, α-chlorohydrin, chlorohydroxypropanone, male infertility, ornidazole, rat

## Introduction

Known chlorinated antifertility compounds that inhibit sperm glycolysis as they reside in the epididymis provide rapid and reversible infertility, but new compounds need to be tested that do not cause side-effects at the higher doses required for toxicity testing (Jones & Cooper, 1999). Ornidazole (ORN) and α-chlorohydrin (ACH) are well known post-testicular antifertility compounds (Coppola & Saldarini, 1974; Jones, 1978; McClain & Downing, 1988a, b). Their administration to male rats leads to reversible infertility

Correspondence: Dr T. G. Cooper, Institute of Reproductive Medicine, Domagkstrasse 11, D-48129 Munster, Germany

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owing to inhibition of the sperm enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) (Ford & Harrison, 1983; Oberländer *et al.*, 1996). As a consequence, hyperactivation of spermatozoa does not occur, so that they cannot penetrate oocytes in vitro, as shown for rats after administration of ORN (Bone *et al.* 2000).

The conversion of ORN to 3-chlorolactate in rats (Jones & Cooper, 1997) suggests a contraceptive action similar to that of ACH, which is thought to act after conversion by a cytoplasmic sperm enzyme glycerol dehydrogenase (Jones & Stevenson, 1983; Stevenson & Jones, 1985) to the active inhibitor of GAPDH (*S*)-3-chlorolactaldehyde. This shares the same stereo-chemistry as the natural substrate of the enzyme (*R*)-glyceraldehyde 3-phosphate, but lacks a transferable phosphate group, with the result that it binds to, but cannot be metabolized by, GAPDH, which is thus unable to metabolize its natural substrate. Inhibition of glycolytic enzymes by ACH is stereo-specific, with only the (*S*)-enantiomer displaying the enzyme inhibitory and antifertility action (Jackson & Robinson, 1976; Ford *et al.*, 1977; Jackson *et al.*, 1977; Jones & Ford, 1984). As ORN is a racemic mixture, metabolic cleavage of the nitroimidazole ring in vivo is anticipated to produce both (*S*) and (*R*) forms of the chlorinated side chain. Surprisingly, both stereo-isomers reduce the motility of rat spermatozoa in vitro (Bone *et al.*, 1997), signifying a different mechanism of motility inhibition from that occurring in vivo.

The present work has examined the nature of the inhibition of sperm GAPDH in vitro by racemic ORN and pure enantiomers, racemic ACH and a putative antifertility agent 1-chloro-3-hydroxypropanone (CHOP), the chlorinated analogue of dihydroxyacetone phosphate, which is converted by porcine sperm TPI stereo-specifically to the GAPDH inhibitor (*S*)-3-chlorolactaldehyde (Jones *et al.*, 1986). CHOP is anticipated to be a more effective inhibitor of sperm GAPDH than is ACH as the inhibitor should be liberated directly on the fibrous sheath by TPI in the same enzyme complex as GAPDH.

## Materials and methods

### *Chemicals and animals*

Chemicals were from Sigma (Deisenhofen, Germany) and were the purest grade available. Male (350–550 g) rats (Charles River, Sulzfeld, Germany) were maintained under 12 h light : 12 h dark (lights on 0700 h) at 22 °C and 50% relative humidity and had access to standard rat chow (Altrumin GmbH, Lage, Germany) and water *ad libitum*. ACH was purchased from Sigma (Deisenhofen, Germany) and distilled twice under vacuum to a purity of 99.9%. Enantiomers of chemically pure (*R*)- and (*S*)-ORN with enantiomeric excesses of 98% and >98%, respectively, were prepared by Prof. G. Haufe, Institute of Organic Chemistry of the University of Münster (Skupin *et al.*, 1997). CHOP was produced by acid hydrolysis of its dimethylketal (pre-CHOP) immediately before the sperm incubation, producing methanol as a by-product. Pre-CHOP (purity 99.9%) was a generous gift from Prof. A. R. Jones (Department of Biochemistry of the University of Sydney, Australia) and synthesized as described by Jones *et al.* (1986).

### *Collection of spermatozoa*

Sperm were obtained from the distal cauda epididymis of rats by cannulating the vas deferens, cutting the mid-cauda epididymis and flushing the luminal contents out with equilibrated medium G, which contains glucose as the sole energy source (Cooper *et al.*, 1996, osmolality: 310 ± 5 mmol/kg). Spermatozoa were collected in 1 mL equilibrated medium G (37 °C, 5% $CO_2$) and were kept for at least 10 min in the incubator. After dispersion of cauda epididymal contents, sperm concentration was estimated by nephelometry at 405 nm (Spectromax 250, Molecular Devices, Sunnyvale, CA, USA) against a standard curve constructed by volumetric determination in a Neubauer improved chamber. Twenty microlitres of the sperm suspension were diluted to 400 μL with phosphate buffered saline for assessment of sperm concentration. In order to determine whether the observed effects were mediated by components of the epididymal fluid present in the sperm incubations, spermatozoa were either used unwashed or washed through Ficoll (see below). In some cases, sonication was used to disrupt the spermatozoa before incubation (see below).

### *Washing of rat spermatozoa*

Rat epididymal luminal fluid in the flushed out contents diluted in medium G was removed by centrifuging 1 mL suspension through 5 mL 5% (w/v) Ficoll in medium G at 1000 ***g*** for 7 min at room temperature (Heraeus, Hanau, Germany). After aspirating the supernatant, Ficoll was removed by resuspending the pellet in 5 mL medium G and centrifuging at 500 ***g*** for 7 min at room temperature (Ole-Dich, Hvidovre, Denmark).

### *Incubation of spermatozoa with (S)-, (R)-, (R,S)-ORN, (R,S)-ACH, CHOP, pre-CHOP or MeOH*

An amount of 800 μL rat sperm suspension ($24 \times 10^6$/mL) was diluted in the same volume of medium G containing (*R,S*)-ACH (0–200 mmol/L), (*R,S*)-ORN (0–20 mmol/L), (*R*)- or (*S*)-ORN (0 or 20 mmol/L), CHOP (0–20 mmol/L) or pre-CHOP (20 mmol/L) and incubated for 2 h at 37 °C in 5% (v/v) $CO_2$. As 2 mol of MeOH are liberated from pre-CHOP per mole of CHOP, MeOH (20 mmol/L) was incubated with spermatozoa as a control. Addition of ACH, ORN and CHOP required lowering the NaCl content of medium G to maintain osmolality at 305–315 mmol/kg. Final sperm concentrations were $12 \times 10^6$/mL.

*Measurement of sperm kinematics*

At the end of incubation before washing the cells, a 4-min video-recording was made for assessment of kinematics by computer-aided sperm analysis (HTM-IVOS: Version 10.6h, Hamilton-Thorne, Beverly, Massachusetts, USA) with the settings for CASA analysis of sperm kinematics as defined by Bone *et al.* (1997).

*Measurement of rat sperm vitality*

Rat sperm vitality was measured by incubating spermatozoa with 5 μg/mL propidium iodide (PI) for 5 min in the dark and monitoring the percentage of cells (>10 000 counted) that exclude the dye by flow cytometry (Epics XL, Coulter, Krefeld, Germany).

*Measurement of enzyme activity in spermatozoa*

At the end of incubation, sperm cell suspensions (1 mL) were washed through 5 mL 5% Ficoll in medium G (2000 ***g***, 7 min, 4 °C), removing most of the supernatant by aspiration to leave the pellet in 240 μL to which 460 μL sonication buffer (100 mmol/L triethanolamine, 5 mmol/L EDTA-$Na_2$, 1 mmol/L DTT, pH 7.3) were added. The resulting suspension was sonicated three times for 7 sec at the highest intensity with a 5-mm probe tip (KLN-Ultraschall-generator, Heppenheim, Germany). Samples were cooled on ice between sonication. An amount of 100 μL sonicate was diluted five-fold in sonication buffer and the sperm concentration again assessed by nephelometry to obtain the basis for enzyme activity. The glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH: EC 1.2.1.12), hexokinase (HK: EC 2.7.1.1) and triosephosphate isomerase (TPI: EC 5.3.1.1) in sperm sonicates were measured by spectrophotometry as described by Bergmeyer (1974) and Ford *et al.* (1981). Methods were modified for a 96-well plate format using 25 or 50 μL extracts. GAPDH and HK activities were assessed on undiluted sonicates, whereas TPI was measured after five-fold dilution of the sonicate. Assayed activities were linear with time and volume of extracts assayed (not shown).

*Repeated washing of the sperm pellet to remove the inhibitor from the active site of GAPDH after incubation with α-chlorohydrin*

Rat spermatozoa suspended in medium G at $24 \times 10^6$/mL were incubated in 2.1 mL medium G containing 0 or 20 mmol/L ACH at 37 °C and 5% $CO_2$. After 2 h, 4 mL suspension was centrifuged through 5 mL Ficoll (Heraeus: 1000 ***g***, 7 min, 4 °C) and the supernatant was removed to leave the pellet in 240 μL to which 460 μL sonication buffer was added before the sperm concentration was measured and the cells sonicated (6 × 7 sec at highest intensity on ice). An amount of 200 μL whole sonicate was removed and the remainder was centrifuged at 20 000 ***g*** for 7 min at 4 °C (Ole-Dich, Hvidovre, Denmark) to separate the soluble (supernatant) from bound (pelleted) GAPDH. The supernatant was retained and the pellet was washed twice by addition of the same volume of sonication buffer before recentrifugation as above. The pellet was sonicated once (5 sec, as above) to solubilize the pellet and GAPDH and TPI were measured as above separately in the whole sonicate as well as in the soluble and in the pelleted fractions.

*Inhibition of rat spermatozoa sonicates by ACH and CHOP*

Rat epididymal spermatozoa were obtained by flushing and washing as above but were centrifuged for 7 min at 1000 ***g*** at 4 °C. The supernatant was removed leaving the sperm pellet in 500 μL to which 500 μL medium G was added immediately before sonication as described above. Spermatozoa were diluted to $24 \times 10^6$/mL and to 500 μL were added 500 μL medium G containing 0–200 mmol/L (*R,S*)-ACH, 0–20 mmol/L CHOP, 20 mmol/L pre-CHOP or 20 mmol/L MeOH and the tubes then incubated for 2 h at 37 °C in 5% $CO_2$. At the end of incubation, sperm concentration was again measured and enzyme activities were measured as above.

*Statistics*

For each sample, kinematic parameters were expressed as the median of all sperm tracks per sample. The data in the text and graphs are the mean values from all males in each treatment group. Dose-dependent comparison of the effect of ORN on kinematic parameters (Fig. 1) were made using one-way analysis of variance (ANOVA) (for GAPDH, TPI, HK, MOT) followed by Dunnet's comparison against the control or one-way ANOVA on ranks (VAP, VCL, VSL, LIN) followed by Dunn's test. One-way ANOVA, followed by Dunnett's method of comparison against controls, was performed for effects of the enantiomers of ORN against drug-free controls (Fig. 2). Comparison between different doses of CHOP and ACH in intact cells (Fig. 3) was made by two-way repeated measures ANOVA and differences between treatment established by Tukey's test. Comparison of sonicates and intact cells (Fig. 4) employed two-way ANOVA on arcsin, square-root transformed percentages followed by Tukey's test. Linear regression analysis was performed on enzyme activities and percentage motility and kinematic parameters.

## Results

*Effects of ornidazole on motility and glycolytic enzymes of intact distal cauda epididymal rat spermatozoa in vitro*

After 2-h incubation in racemic ORN, the percentage of motile spermatozoa was not altered, but the kinematic parameters VSL and LIN were significantly reduced at 10 mmol/L. Of the enzymes measured, only GAPDH was inhibited to 77 and 45% at 1 and 10 mmol/L, respectively (Fig. 1). In a further experiment, spermatozoa were

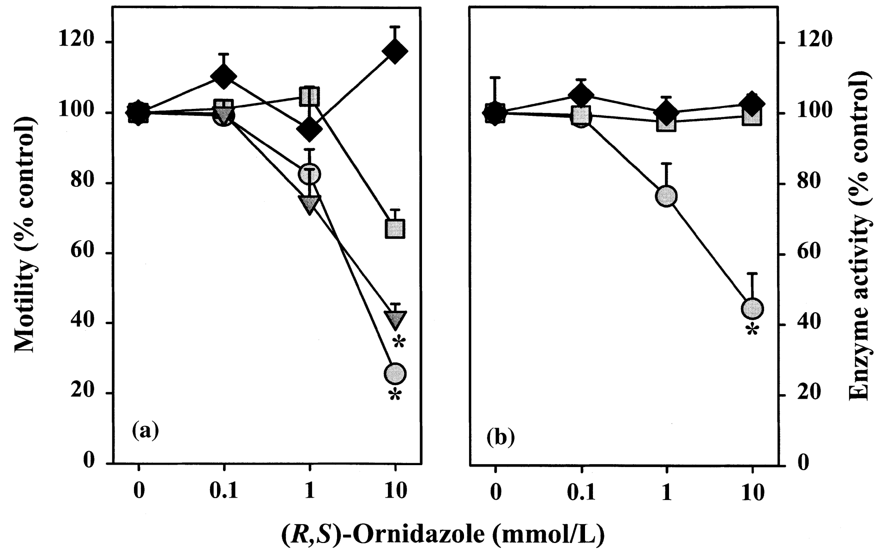


**Figure 1.** (a) Motility (◆) and kinematic parameters (ordinate: VSL (●), VCL (■), LIN (▼)) and (b) the activity of glycolytic enzymes (ordinate: GAPDH (●), TPI (■), HK (◆)) of spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$/mL in medium G containing increasing concentrations of (*R*,*S*)-ORN (abscissa). Values are expressed as a percentage of control (mean + SEM, *n* = 4). Control values (in absence of ORN) were VCL 46.2 ± 2.6 μm/sec; VCL 87.5 ± 1.7 μm/sec; LIN 53.5 ± 2.6%; MOT 51.4 ± 6.8%; GAPDH 4.71 ± 1.37 mU/$10^6$ spermatozoa; TPI 46.03 ± 8.67 mU/$10^6$ spermatozoa; HK 2.96 ± 0.08 mU/$10^6$ spermatozoa. *Significantly different from drug-free control.

incubated with pure enantiomers of 10 mmol/L ORN. Both the enantiomers were as effective as the racemic mixture in inhibiting GAPDH activity (≈30% of control) and kinematic parameters (VSL: ≈30%, VCL: ≈60% and LIN: ≈50% of control) (Fig. 2) but had no effect on vitality.

*Effects of ACH and CHOP on motility and glycolytic enzymes of intact distal cauda epididymal rat spermatozoa in vitro*

Percentage motility was unchanged up to 10 mmol/L ACH and a few spermatozoa were motile at 100 mmol/L, whereas CHOP rendered spermatozoa completely immotile at this concentration (Fig. 3a). Methanol (20 mM), that is necessarily present in the CHOP-containing medium as a result of hydrolysis of its precursor, did not alter the percentage motility of rat spermatozoa (Table 1).

Neither ACH nor CHOP had an effect on kinematic parameters at 0.1 mmol/L, but above this dose there were dose-dependent decreases in VSL (Fig. 3b), VCL (Fig. 3c), VAP (Fig. 3d) and LIN (Fig. 3e) as the concentration of ACH was increased. At 1 mmol/L ACH induced significant reductions in VSL (46%), VCL (22%), and LIN (43%) and further reductions were noted at 10 mmol/L, with close to immotility occurring with 100 mmol/L. A reduction in ALH was only observed beyond 10 mmol/L ACH (Fig. 3f).

As was found for sperm motility, the effect on kinematic parameters was more marked for CHOP than ACH at high doses. Dose-dependent reductions in VSL, VAP and LIN from 0.1 to 10 mmol/L CHOP differed from the maintenance of VCL at 1 mmol/L at the levels of drug-free controls (Fig. 3c) and the increase to 150% of control values

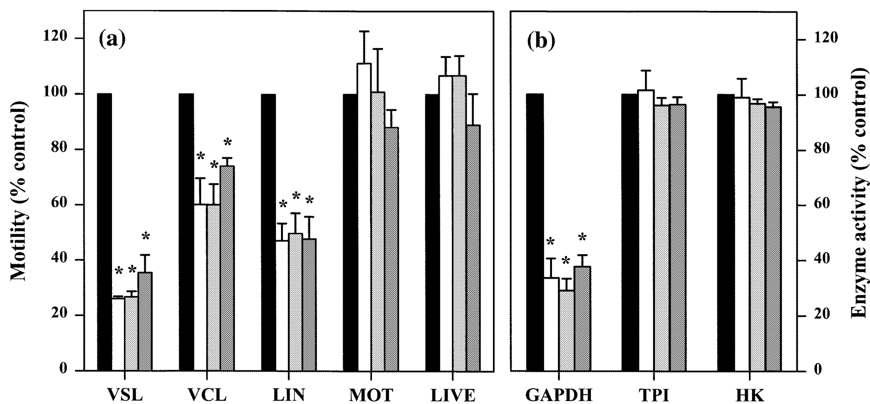


**Figure 2.** (a) Kinematic parameters (VSL, VCL, LIN), percentage of motile spermatozoa (MOT), percentage of vital spermatozoa (live) and (b) the activity of glycolytic enzymes (GAPDH, TPI, HK) in spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$/mL in medium G containing no ORN (black bars), 10 mmol/L (*R*,*S*)-ORN (white bars), 10 mmol/L (*R*)-ORN (light grey bars) or 10 mmol/L (*S*)-ORN (dark grey bars). Values are expressed as a percentage of control (mean + SEM, *n* = 3). Control values (in absence of ORN) were VSL 45.4 ± 0.9 μm/sec; VCL 85.4 ± 1.2 μm/sec; LIN 53.7 ± 1.2%; MOT 46.4 ± 14.6%; VIA (viability) 44.1 ± 12.8%, GAPDH 6.011 ± 0.197 mU/$10^6$ spermatozoa; TPI 53.300 ± 2.443 mU/$10^6$ spermatozoa; HK 3.091 ± 0.130 mU/$10^6$ spermatozoa. *Significantly different from drug-free control.

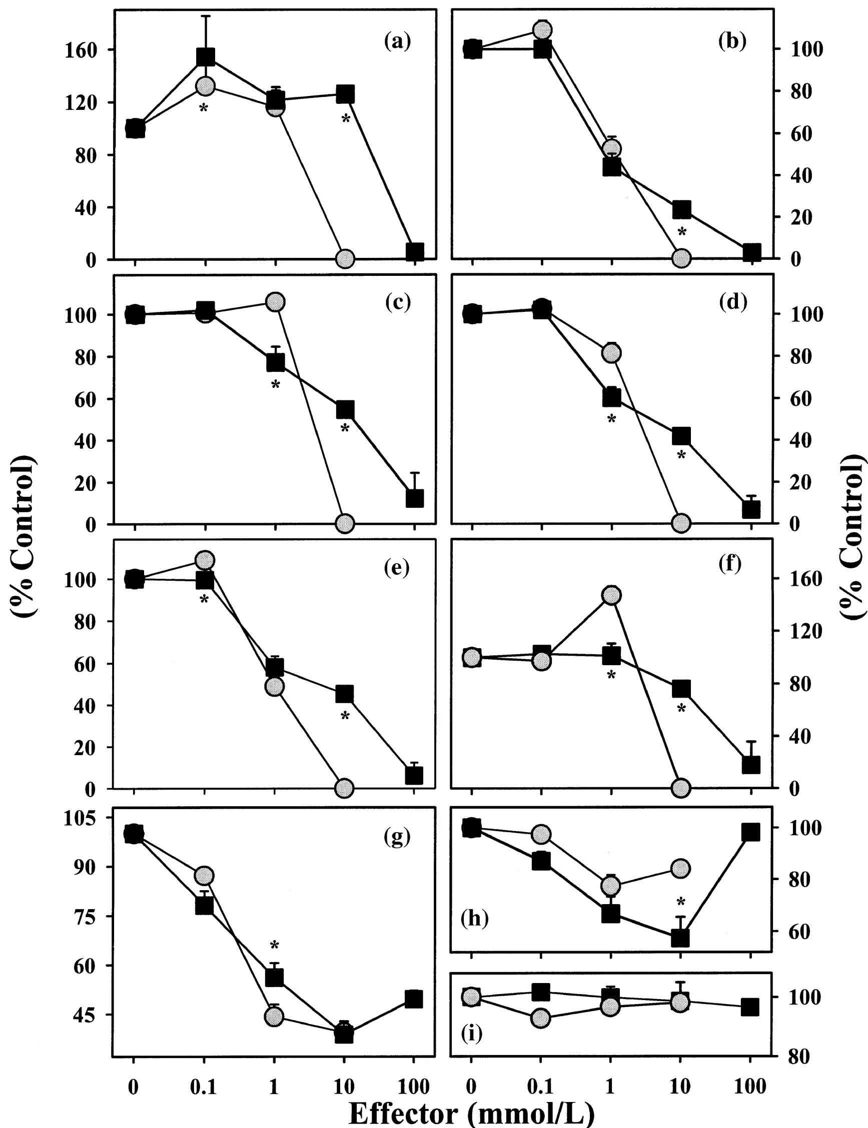


**Figure 3.** Kinematic parameters ((a) MOT, (b) VSL, (c) VCL, (d) VAP, (e) LIN, (f) ALH) and the activities of glycolytic enzymes ((g) GAPDH, (h) TPI, (i) HK) in spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$/mL in medium G containing increasing concentrations ACH (■) or CHOP (◯). Values are expressed as a percentage of control (mean + SEM; ACH $n = 4$; CHOP $n = 7$). Control values (in absence of ACH) were VSL 57.9 ± 4.9 μm/sec; VCL 99.3 ± 3.9 μm/sec; VAP 64.5 ± 7.0 μm/sec; LIN 59.8 ± 2.1%; MOT 31.4 ± 4.1%; GAPDH 7.65 ± 0.39 mU/$10^6$ spermatozoa; TPI 36.71 ± 5.41 mU/$10^6$ spermatozoa; HK 3.22 ± 0.22 mU/$10^6$ spermatozoa. Control values (in absence of CHOP) were VSL 50.4 ± 3.8 μm/sec; VCL 86.5 ± 1.4 μm/sec; VAP 58.2 ± 2.8 μm/sec; LIN 54.8 ± 3.5%; MOT 39.2 ± 5.6%; GAPDH 6.70 ± 0.55 mU/$10^6$ spermatozoa; TPI 53.30 ± 5.18 mU/$10^6$ spermatozoa; HK 1.87 ± 0.29 mU/$10^6$ spermatozoa. *Significant difference between drugs at the same dose.

in ALH (Fig. 3f). These differences were responsible for the significant differences between the effects of the chlorinated compounds at 1 mmol/L on VCL, VAP and ALH. By contrast, methanol (20 mmol/L) did not influence kinematic parameters (Table 1).

Over the range 0.1–10 mmol/L there were dose-dependent decreases in GAPDH by both ACH and CHOP. GAPDH and TPI were inhibited at 0.1 mmol/L ACH and maximal inhibition (GAPDH 39%, TPI 57%) was found at 10 mmol/L. One hundred mmol/L ACH did not reduce TPI activity and inhibited GAPDH less than at 10 mmol/L. Differences between the enzyme inhibition by ACH and CHOP were found with CHOP being the more effective inhibitor of GAPDH at 1 mmol/L and ACH being the better inhibitor of TPI at 10 mmol/L. No inhibition of HK activity was observed at any concentration of either compound (Fig. 3). Washing spermatozoa free of epididymal fluid before incubation to remove epididymal fluid did not affect the inhibitory activity of ACH (data not shown). Methanol (20 mmol/L) did not effect any enzyme activities (Table 1).

*Effect of ACH on the vitality of intact rat spermatozoa*

After 2-h incubation in medium G followed by incubation with propidium iodide to label dead cells, the percentage of viable cells estimated by flow cytometry was 48.6 ± 8.1%, which accords with the motility of 49.2 ± 9.3%. Similar agreements were found when spermatozoa from the same suspension were incubated in 0.1, 1 and 10 mmol/L ACH (motilities 33.0–36.9% and 36.0–44.4% viable cells). At 100 mmol/L (*R,S*)-ACH the percentage of vital cells decreased to 1.8%, reflecting the lack of motility (0%). The concentration of ACH did not influence the pH of the medium, which remained in the range 7.35–7.30 at the end of incubation.

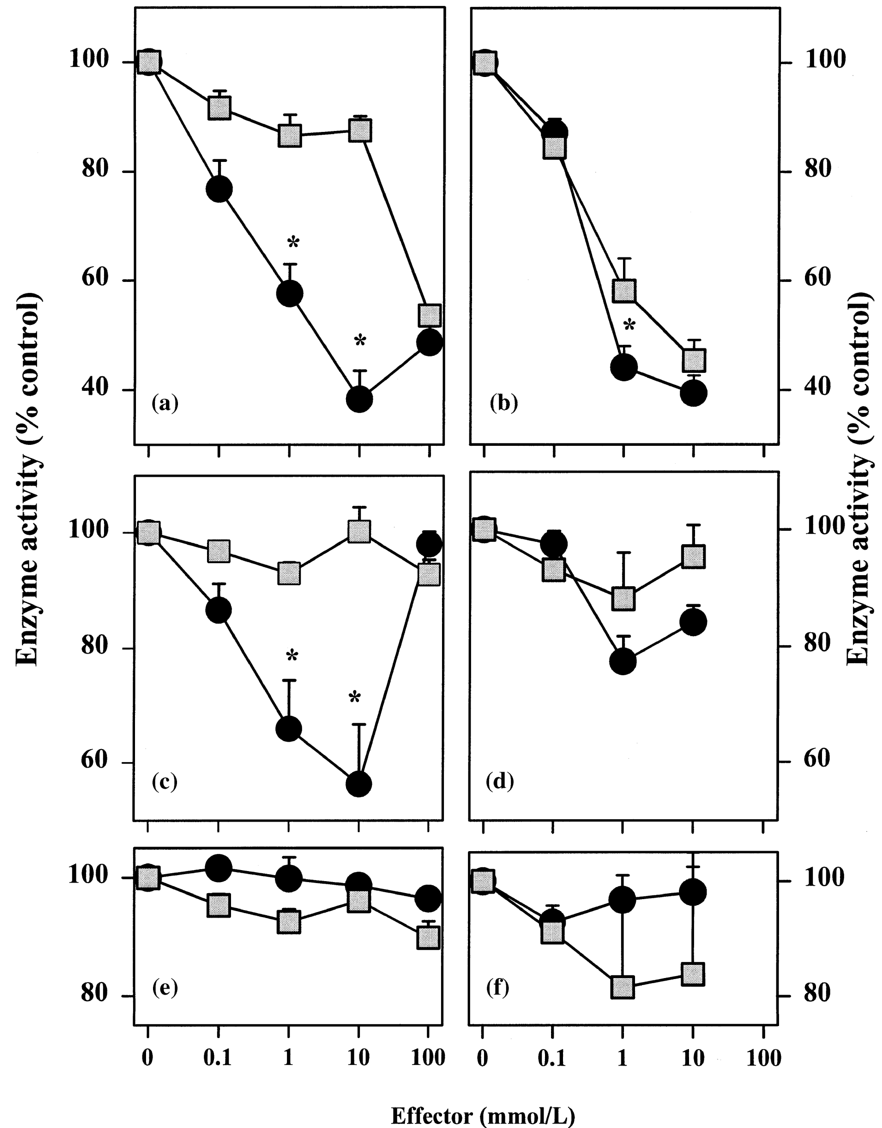


**Figure 4.** Activities of glycolytic enzymes ((a, b) GAPDH, (c, d) TPI and (e, f) HK) in suspensions (●) or sonicates (□) of spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$/mL in medium G containing ACH (a, c, e) or CHOP (b, d, f). Values are expressed as a percentage of control (mean ± SEM; ACH: $n = 4$, CHOP suspension: $n = 7$, sonicates: $n = 5$). Control values (in absence of ACH) were: GAPDH 7.65 ± 0.39 mU/$10^6$ spermatozoa; TPI 36.71 ± 5.41 mU/$10^6$ spermatozoa; HK 3.22 ± 0.22 mU/$10^6$ spermatozoa (suspensions), GAPDH 2.61 ± 0.31 mU/$10^6$ spermatozoa; TPI 65.35 ± 8.93 mU/$10^6$ spermatozoa; HK 4.00 ± 0.27 mU/$10^6$ spermatozoa (sonicates). Control values (in absence of CHOP) were: GAPDH 6.70 ± 0.55 mU/$10^6$ spermatozoa; TPI 53.30 ± 5.18 mU/$10^6$ spermatozoa; HK 1.87 ± 0.29 mU/$10^6$ spermatozoa (suspensions), GAPDH 4.57 ± 0.45 mU/$10^6$ spermatozoa; TPI 62.90 ± 5.58 mU/$10^6$ spermatozoa; HK 1.94 ± 0.38 mU/$10^6$ spermatozoa (sonicates). *Significant difference between cell preparations at the same dose.

*Nature of inhibition of GAPDH and TPI in spermatozoa from the distal cauda epididymis by ACH*

After 2-h incubation of intact spermatozoa with ACH and separation of soluble and insoluble sperm fractions by sonication and centrifugation, only 2.5% of the GAPDH activity was found in the soluble fraction and more than 95% was pelletable. A similar distribution was found for TPI (6.1% soluble) and the distribution of neither enzyme in this fraction was influenced by incubation for 2 h in 10 mmol/L ACH (3.9 and 5.3%, respectively, Table 2). The inhibition of GAPDH by ACH after incubation of the intact cells followed by sonication was 56.1% and after washing the pellet twice the remaining activity was still 60% of controls. Likewise, the inhibition of TPI by ACH (47.4%) remained unchanged after washing the pellet (49.5%) (Table 2).

*Inhibition of glycolytic enzymes in sonicated spermatozoa from the distal cauda epididymis by ACH and CHOP*

In contrast to intact cell suspensions, sonicated preparations of rat spermatozoa incubated in the presence of 0.1–10 mmol/L ACH did not suffer reductions in GAPDH or TPI activities. With 100 mmol/L ACH, the inhibition of GAPDH in incubated sonicates was similar to that of the intact suspensions (Fig. 4a). In contrast, CHOP inhibited the GAPDH activity of incubated sonicates to the same extent as the cell suspensions, whereas TPI and HK were not inhibited (Fig. 4b). Neither pre-CHOP (10 mmol/L) nor MeOH

**Table 1.** Kinematic parameters and the activities of glycolytic enzymes of spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$ spermatozoa/mL in medium G containing methanol or pre-CHOP

| | Kinematic parameters and motility | | | | Enzyme activities | | |
|---|---|---|---|---|---|---|---|
| Compound | VSL | VCL | LIN | MOT | GAPDH | TPI | HK |
| 20 mmol/L MeOH | 114.1 ± 8.9 | 98.5 ± 3.6 | 113.7 ± 6.3 | 76.9 ± 21.3 | 96.3 ± 13.5 | 115.8 ± 18.6 | 111.1 ± 16.0 |
| 10 mmol/L pre-CHOP | 87.3 ± 2.1 | 96.5 ± 2.5 | 90.7 ± 1.4 | 81.3 ± 18.4 | 108.4 ± 5.9 | 106.9 ± 7.2 | 96.2 ± 12.8 |

Values are expressed as percentages of controls (mean ± SEM, $n = 4$).

**Table 2.** Absolute values of the activities of GAPDH and TPI in whole sonicates, supernatants and the pellets of spermatozoa from the distal cauda epididymis after incubation for 2 h at a concentration of $12 \times 10^6$ spermatozoa/mL in 10 mmol/L distilled (*R*,*S*)-ACH

| | GAPDH (mU/$10^6$ spermatozoa) | | | TPI (mU/$10^6$ spermatozoa) | | |
|---|---|---|---|---|---|---|
| Fraction | Control | ACH | % Control | Control | ACH | % Control |
| Homogenate | 6.47 ± 1.28 | 2.48 ± 0.56* | 43.9 | 45.11 ± 5.56 | 23.74 ± 1.47* | 52.6 |
| Supernatant | 0.20 ± 0.02 | 0.13 ± 0.01* | 65.0 | 3.00 ± 0.57 | 1.30 ± 0.33* | 43.3 |
| Pellet | 7.95 ± 1.66 | 3.18 ± 0.83* | 40.0 | 46.39 ± 6.28 | 23.44 ± 2.19* | 50.5 |
| Total | 8.15 ± 1.68 | 3.31 ± 0.84* | 40.6 | 49.39 ± 6.85 | 24.74 ± 2.52* | 50.1 |

Values are mean ± SEM ($n = 4$); *$p < 0.05$, compared with control values.

**Table 3.** Activities of glycolytic enzymes of sonicates of spermatozoa from the distal cauda epididymis after 2-h incubation at a concentration of $12 \times 10^6$ spermatozoa/mL in medium G containing methanol or pre-CHOP

| | Enzyme activity | | |
|---|---|---|---|
| Compound | GAPDH | TPI | HK |
| 20 mmol/L MeOH | 94.4 ± 11.0 | 108.7 ± 5.5 | 101.5 ± 11.8 |
| 10 mmol/L pre-CHOP | 94.4 ± 9.2 | 105.0 ± 4.8 | 124.3 ± 28.7 |

Values are expressed as a percentage of control (mean ± SEM, $n = 3$).

(20 mmol/L) had an effect on enzyme activities on sonicated sperm preparations (Table 3).

*Relationship between extent of enzyme inhibition and kinematics of spermatozoa from the distal cauda epididymis*

Linear regression revealed significant correlations between the activity of GAPDH measured at the end of 2 h incubation and the kinematic parameters displayed at that time (Table 4). Correlations were weak and not significant between GAPDH activity and that of percentage motility (<0.119), TPI (<0.395) or HK (<0.263), or between kinematic parameters and TPI and HK. Of particular note were the highly significant correlation coefficients ($R > 0.6$) between GAPDH activity and many kinematic parameters for all three compounds. For VCL and ALH the relationship depended on which chloro-compound was present during incubation; as GAPDH activity declined, VSL, VCL and ALH also declined when ACH was present, whereas in the presence of CHOP, VSL declined, VCL did not change and ALH increased. Inhibition by ORN was more like that of ACH in that only positive correlations

**Table 4.** Correlation coefficients calculated from linear regression analysis of GAPDH activity and the percentage motility and kinematic parameters of intact spermatozoa from the distal cauda epididymis incubated for 2 h at a concentration of $12 \times 10^6$ spermatozoa/mL with (*R*,*S*)-ORN, (*R*,*S*)-ACH and CHOP

| Parameter | ORN | ACH | CHOP |
|---|---|---|---|
| VSL | +0.691* | +0.811* | +0.661* |
| VAP | +0.657* | +0.795* | +0.581* |
| VCL | +0.546* | +0.779* | −0.505* |
| LIN | +0.667* | +0.487* | +0.701* |
| STR | +0.639* | +0.785* | +0.743* |
| WOB | +0.636* | +0.653* | +0.654* |
| ALH | +0.266 | +0.787* | −0.715* |
| BCF | +0.309 | +0.515* | +0.312 |
| MOT | +0.048 | +0.028 | +0.119 |

* Significant correlation.

between GAPDH activity and kinematic parameters were found (Table 4).

## Discussion

In this study the effects of (*R*)- and (*S*)-ORN on rat sperm motility and glycolytic enzymes were examined in order to understand the unexpected findings reported by Bone *et al.* (1997), that (*R*)- and (*S*)-ORN decrease, to the same extent, sperm kinematics in medium with glucose as the only substrate. The equal inhibition of motility parameters by (*R*)- and (*S*)-ORN, as well as the racemic mixture, confirmed the report of Bone *et al.* (1997); in addition, all three compounds were shown to depress GAPDH activity to the same extent. The inhibition of GAPDH by (*R*)-ORN, which should not give rise to (*S*)-3-chlorolactaldehyde if ACH is liberated, was unexpected. This highlights a difference from in vitro inhibition of GAPDH activity by ACH, which is stereo-specific (Jones & Ford, 1984; Stevenson & Jones, 1985). The metabolites liberated from ORN and their stereo-chemistry are not known, but unlike amino-chloropropanol (Jones *et al.*, 1979), scission of the C–N bond of ORN is not inhibited by a monoamine oxidase inhibitor (Jones & Cooper, 1997), which may indicate another mechanism of cleavage favouring liberation of an (*S*)-enantiomeric form. The cleavage of the C–N bond to yield a non-chiral compound that is hydrolysed to CHOP and subsequently to 3-chlorolactadehyde, for example, would explain the positive inhibition by both (*R*)- and (*S*)-ORN. Thus, ORN may offer a route to the putative active intermediate that does not include ACH and could therefore be free of some of its toxic side-effects, justifying further research into the metabolism of ORN.

The possibility that the inhibition of sperm motility and enzyme activity caused by both ORN enantiomers is mediated by the nitroimidazole ring, rather than a metabolite from the chlorinated side-chain, is unlikely, as 5-nitro-3-methyl-imidazole has no effect on rat sperm motility in vitro (Bone *et al.*, 1997). Another difference of the in-vitro findings observed here from those reported after feeding ORN (Oberländer *et al.*, 1996) is the lack of inhibition of sperm TPI. There are presumably differences between the conversion in vivo (Jones & Cooper, 1997) and in vitro. The inhibition of GAPDH and TPI after incubation of intact rat spermatozoa with ACH agrees with the inhibition demonstrated in ejaculated ovine (Brown-Woodman *et al.*, 1978) and epididymal porcine spermatozoa (Stevenson & Jones, 1985).

The unexpected observation that 100 mmol/L ACH failed to inhibit the activity of GAPDH and TPI in intact spermatozoa as much as 10 mmol/L, may be explained by such enzyme inhibition only being possible in living spermatozoa in which metabolism of ACH to (*S*)-3-chlorolactaldehyde can occur, so that sperm preparations containing a high proportion of dying cells would respond less to glycolytic inhibitors. Indeed, the flow cytometry demonstrated propidium iodide staining of almost all rat spermatozoa incubated for 2 h in 100 mmol/L ACH, suggesting toxicity of this dose under the incubation conditions employed.

The nature of the enzyme inhibition was further explored by taking advantage of observations that sperm GAPDH is not cytosolic, but is bound to the fibrous sheath and is thus pelleted after sonication and centrifugation (Westhoff & Kamp, 1997). As the inhibition of GAPDH and TPI in the pelleted fraction of sonicated spermatozoa remained after washing the flagella free from loosely bound or soluble inhibitors, the inhibition is deemed to be not easily reversible. The lack of inhibition of GAPDH when sonicated preparations of rat spermatozoa were incubated with ACH (up to 10 mmol/L) is in agreement with its failure to inhibit the activity of GAPDH present in ovine sperm extracts (Mohri *et al.*, 1975). Thus, ACH has to be metabolized into an active inhibitor of GAPDH inside rat spermatozoa as it does in the boar (Jones & Stevenson, 1983; Stevenson & Jones, 1985). The comparable inhibitory effects of spermicidal concentrations of ACH (100 mmol/L) on GAPDH in sonicated and non-sonicated spermatozoa could be due to binding of ACH itself to the active centre of GAPDH, but with a much lower affinity than the active inhibitor in intact cells.

CHOP, the chlorinated analogue of dihydroxyacetone phosphate, has a similar action on rat spermatozoa as ACH, but is more effective, even allowing for the fact that racemic ACH was used so that one half of the concentration of active (*S*)-ACH was present. Immotility occurred with CHOP at 10 mmol/L, but it did not block TPI activity of rat epididymal spermatozoa in vitro as much as did ACH. Triosephosphate isomerase activity from epididymal spermatozoa from the ram and pig is reduced by CHOP (Jones, 1987; Jones & Cooney, 1987), but in those studies only the soluble TPI activity was estimated. As CHOP is both the substrate for, and an inhibitor of, TPI, too drastic an inhibition would have the consequence that the primary inhibitor of GAPDH (*S*)-3-chloro-lactaldehyde, would not be generated; thus some remaining TPI activity is necessary for the antiglycolytic action of CHOP. The fact that the dimethyl ketal of CHOP (pre-CHOP) was ineffective suggests that spermatozoa are unable to hydrolyse the precursor, although this occurs after oral ingestion (Cooper & Jones, 2000). Confirmation that the inhibitory effects on sperm motility and glycolytic enzymes are specific to CHOP is further strengthened by the failure of methanol, liberated from pre-CHOP at twice the molar concentration of CHOP, to affect sperm motility or enzyme activity.

At 0.1 mmol/L the activities of GAPDH and TPI were inhibited by ACH but reductions in kinematic parameters were not observed until 1 mmol/L. This suggests that the decrease in kinematic parameters is a consequence of the reduction in GAPDH and TPI activities below a certain

threshold. GAPDH may be more influential as high correlation coefficients ($R > 0.6$) were only observed between kinematic parameters (VSL, VAP, STR, LIN and WOB) and the activity of GAPDH and not the activity of TPI.

The major difference between the inhibitory actions of ACH and CHOP was the action by the latter on GAPDH activities of sperm sonicates, indicating that CHOP can easily be converted by TPI to (*S*)-3-chlorolactaldehyde without an intact cell or metabolism within the cytoplasm. Upon sonication the cytosolic enzyme and cofactors necessary for the metabolism of ACH are presumably too dilute for effective conversion. As an enzyme complex consisting of TPI, GAPDH and phosphoglycerate kinase (PGK) is bound to the fibrous sheath of spermatozoa (Westhoff & Kamp, 1997) (*S*)-3-chloro-lactaldehyde produced from CHOP by TPI inside this enzyme complex may be a better inhibitor of sperm GAPDH than that generated externally, such as from ACH. This makes CHOP a good compound for investigating the specificity of sperm GAPDH inhibition, which is a prerequisite for the development of a post-testicular and antiglycolytic contraceptive for males.

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