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Inhibition of Fructolysis in Boar Spermatozoa by the Male Antifertility Agent (*S*)-α-Chlorohydrin

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Abstract

The (*S*)-isomer of the male antifertility agent α-chlorohydrin strongly inhibited the oxidative metabolism of fructose by boar spermatozoa *in vitro*. The result of this action, which has been deduced to be an inhibition of glyceraldehydephosphate dehydrogenase, caused an accumulation of fructose-1,6-bisphosphate and the triosephosphates, and a decrease in substrate-level phosphorylation with a concomitant lowering of the energy charge potential of the spermatozoa. The (*R*)-isomer of α-chlorohydrin had no inhibitory activity on fructolysis.

A study of the comparative metabolism of (*R*)-[3-^{36}Cl]-α-chlorohydrin and (*R,S*)-[3-^{36}Cl]-α-chlorohydrin by boar spermatozoa showed that it is the (*S*)-isomer that specifically undergoes a process of oxidative metabolism to (*R*)-3-chlorolactaldehyde. It is proposed that this endogenous oxidation product, which has the same absolute configuration as the substrate for glyceraldehydephosphate dehydrogenase, is the active metabolite of (*S*)-α-chlorohydrin that inhibits this enzyme. Exogenous (*R,S*)-3-chlorolactaldehyde inhibited the oxidative metabolism of fructose by boar spermatozoa, apparently by a mechanism similar to that of (*S*)-α-chlorohydrin.

Introduction

α-Chlorohydrin (3-chloropropan-1,2-diol) satisfies many of the criteria required of an ideal male antifertility agent. When low doses are administered orally to male rats, α-chlorohydrin is apparently non-toxic, has an immediate effect on fertility which ceases when treatment is withdrawn and an action directed specifically at mature spermatozoa (Jones 1978). Studies *in vitro* with mature spermatozoa obtained from the cauda epididymides of the rat, ram, boar and rhesus monkey, and with ejaculated human spermatozoa, have shown that the compound inhibits glycolysis (Homonnai *et al.* 1975; Brown-Woodman *et al.* 1978; Ford *et al.* 1979; Ford and Harrison 1980; Hutton *et al.* 1980). The principle site of this inhibitory action in ram and rhesus monkey spermatozoa is known to involve the glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) reaction (Brown-Woodman *et al.* 1978; Ford and Harrison 1980). This inhibitory activity towards the metabolism of ram and boar spermatozoa *in vitro*, however, is not immediate (Mohri *et al.* 1975; Hutton *et al.* 1980). Evidence of a delay in its action, together with a decrease in the ability of α-chlorohydrin to inhibit glycolysis when it was added to ram spermatozoal preparations which had been subjected to ultrasonic disruption, suggested that the true inhibitor was a metabolite of α-chlorohydrin (Brown-Woodman *et al.* 1978).

The α-chlorohydrin used in all of these experiments was a racemic mixture. When the syntheses of the (*R*)- and (*S*)-isomers were achieved, (*S*)-α-chlorohydrin was

shown to produce the antifertility effect *in vivo* in the rat (Jackson *et al.* 1977), with the (*R*)-isomer being ineffective.

This paper presents data showing the effect of (*S*)-α-chlorohydrin on the fructolytic activity and the energy charge potential of mature boar spermatozoa *in vitro*. The comparative metabolism of (*R,S*)-[3-^{36}Cl]-α-chlorohydrin and (*R*)-[3-^{36}Cl]-α-chlorohydrin by boar spermatozoa *in vitro* has been investigated and evidence is presented that the active inhibitory metabolite of (*S*)-α-chlorohydrin is (*R*)-3-chlorolactaldehyde.

Materials and Methods

Collection of Spermatozoa

For each experiment, entire testis–epididymis complexes were obtained from groups of 15–30 mature boars (*Sus domesticus*) within 1 h of slaughter at the abattoir. Spermatozoa were flushed from the cauda epididymides (Hutton *et al.* 1980) with the phosphate-buffered saline devised by Robinson (1949) with glucose omitted. The spermatozoal suspension (40–60 ml) was centrifuged at 2500 *g* for 10 min at 20°C. The supernatant solution was removed and the sedimented spermatozoa were resuspended in phosphate-buffered saline, centrifuged again at 2500 *g* for 10 min at 20°C and the spermatozoa finally suspended in the same medium at 34°C at a concentration of 2–3 g of sedimented spermatozoa per 10 ml (15–25 mg protein/ml).

Metabolic Studies

For the measurement of metabolically derived $^{14}CO_2$, suspensions of washed spermatozoa, in a final volume of 1 ml, were incubated for varying times at 34°C in Warburg flasks (capacity 27 ml) in the presence of (*S*)-α-chlorohydrin (0, 0·005, 0·01, 0·1, 0·2, 0·3, 0·4, 0·5, 1·0, 5, 10 mM) with the following substrates (0·5 μCi, 18·5 kBq): D-[U-^{14}C]fructose (1 mM), [U-^{14}C]pyruvate (2 mM) and L-[U-^{14}C]lactate (2 mM). The centre well contained 0·1 ml of 2 M NaOH and the flasks were shaken at 120 cycles/min with air as the gas phase. The incubations were terminated by adding 0·1 ml of 3 M $HClO_4$ and the liberated $^{14}CO_2$, trapped in the centre well as $Na_2{}^{14}CO_3$, was measured by liquid scintillation counting (Dawson 1977).

Assay of Glycolytic Intermediates

Suspensions of washed spermatozoa in phosphate-buffered saline in a final volume of 5 ml were incubated at 34°C for 1 h in unsealed conical flasks (capacity 25 ml) with D-fructose (1 mM) as substrate. Each incubation contained either (*S*)-α-chlorohydrin (0, 0·05, 0·1, 0·2, 0·5 mM), or a mixture of (*R*)-α-chlorohydrin (0·5 mM) and (*S*)-α-chlorohydrin (0·5 mM), or (*R,S*)-α-chlorohydrin (1 mM), or (*R*)-α-chlorohydrin (100 mM) or (*R,S*)-3-chlorolactaldehyde (5 mM). At the end of the incubation or, for zero-time samples, before the addition of the spermatozoal suspension, 0·5 ml of 3 M $HClO_4$ was added to each flask. After removal of the deproteinized material by centrifugation at 2500 *g* for 10 min at 4°C, the acid extract was neutralized to pH 6·5–7·5 with 3 M KOH by using universal indicator paper. The neutralized solution was adjusted to a final volume of 6·5 ml with distilled water and kept overnight at − 20°C. The precipitated $KClO_4$ was removed by centrifugation at 2500 *g* for 10 min and the supernatant solution, stored at − 20°C, used for the assay of glycolytic intermediates and adenine nucleotides. Adenine nucleotide assays were carried out as soon as practically possible (within 4 days) to minimize ATP loss.

Fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Michal and Beutler 1974), 3-phosphoglycerate and 2-phosphoglycerate (Czok and Eckert 1963), lactate (Hohorst 1963), pyruvate, ADP and AMP (Adam 1963) and ATP (Lamprecht and Trautschold 1963) were measured spectrophotometrically with a Varian 634 spectrophotometer by following the change in A_{340nm} corresponding to the oxidation or reduction of nicotinamide nucleotides. The total adenine nucleotide content was calculated as: [ATP]+[ADP]+[AMP]. The energy-charge potential was calculated as the ratio

$$([ATP]+\tfrac{1}{2}[ADP])/([ATP]+[ADP]+[AMP])$$

as described by Atkinson (1968).

Uptake and Metabolism of α-Chlorohydrin by Boar Spermatozoa

Suspensions of washed spermatozoa (3 ml) were incubated at 34°C in unsealed conical flasks shaken at 120 cycles/min. At zero time, D-fructose (1 mM) and (*R,S*)-[3-^{36}Cl]-α-chlorohydrin (10–100 μM) were added to the suspension and at various times (0·25 min–2 h), duplicate samples (100 μl) were removed, filtered through Millipore filters (0·22 μm) and the filters washed with phosphate-buffered saline (2 × 5 ml). The air-dried filters were assayed for radioactivity by liquid scintillation counting.

Incubations of washed spermatozoa with D-fructose (1 mM) and either (*R,S*)-[3-^{36}Cl]-α-chlorohydrin (100 μM) or (*R*)-[3-^{36}Cl]-α-chlorohydrin (100 μM) were carried out for 2 h in a similar manner. The suspensions were centrifuged at 2500 *g* for 10 min and the supernatant solutions (A) kept. The pellets were suspended in water (1 ml) and the cells were disrupted by sonic oscillation (20 kHz) for 2 min by using a Branson Sonifier, model B12 (Branson Sonic Power Co., Danbury, Massachusetts, U.S.A.). Cell debris was removed by centrifugation at 2500 *g* for 20 min at 20°C and the supernatant solutions (B) kept. As soon as possible, samples (200 μl) of both supernatant solutions (A and B) from each incubation were applied to separate precoated thin-layer chromatography (t.l.c.) plates of silica gel G (0·5 mm thick) (E. Merck, Darmstadt, Germany). The t.l.c. plates were developed in a solvent system comprising chloroform: methanol (7 : 3 by vol.) and radioactive areas were detected by scanning on a Berthold Radiochromatogram Scanner, model LB2723 (Berthold, Wildbad, Germany). Authentic compounds had R_F values of 0·10 (chloride ion), 0·47 (3-chlorolactaldehyde) and 0·55 (α-chlorohydrin).

Effect of (R,S)*-3-Chlorolactaldehyde on the Oxidation of Fructose*

The procedures were identical to those used for studying the effect of the isomers of α-chlorohydrin on the oxidation of D-[U-^{14}C]fructose. (*R,S*)-3-Chlorolactaldehyde, as a water-insoluble dimer, was converted into the water-soluble monomer by heating it for several minutes at 90°C in water.

As with (*S*)-α-chlorohydrin, solutions of (*R,S*)-3-chlorolactaldehyde were examined in all analytical procedures. Neither compound interfered with any of the assay methods.

Counting of Radioactivity

Liquid scintillation counting was performed in a Nuclear Chicago Isocap/300 liquid scintillation counter, counts per minute being corrected for background and converted to disintegrations per minute by reference to external standard quench-correction curves prepared from a ^{14}C-labelled standard and an arbitrary ^{36}Cl standard. The scintillation fluid (5 ml per vial) consisted of 0·6% (w/v) 2,5-diphenyloxazole (Koch-Light Ltd., Colnbrook, Bucks., U.K.) in toluene–Triton X-100 (1 : 1 v/v).

Materials

(*R,S*)-Glycidaldehyde and (*R,S*)-α-chlorohydrin were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. The (*R,S*)-α-chlorohydrin was purified by two distillations at reduced pressure (b.p. 108°C at 3 mm). (*R*)- and (*S*)-α-Chlorohydrin were synthesized by the method of Porter and Jones (1982). (*R,S*)-[3-^{36}Cl]-α-Chlorohydrin (sp. act. 240 μCi/mmole) was prepared according to Jones *et al.* (1978), and (*R*)-[3-^{36}Cl]-α-chlorohydrin (sp. act. 240 μCi/mmole) from (*R*)-glycidol (Baldwin *et al.* 1978) and H^{36}Cl gas (liberated from 0·1–0·3 M H^{36}Cl) by the same procedure. (*R,S*)-3-Chlorolactaldehyde was synthesized from glycidaldehyde according to Williams *et al.* (1960). D-[U-^{14}C]Fructose, L-[U-^{14}C]lactate, [U-^{14}C]pyruvate and 0·1–0·3 M H^{36}Cl were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. All enzymes, substrates and coenzymes were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. All other chemicals and reagents were of analytical grade and all solutions were prepared in glass-distilled deionized water.

Protein Determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Results were calculated using the linear transform equation of Coakely and James (1978).

Results

Inhibition of the Oxidation of Fructose

Low concentrations of (*S*)-α-chlorohydrin inhibited the production of respiratory CO_2 by boar spermatozoa with D-[U-^{14}C]fructose (1 mM) as substrate (Table 1). When L-[U-^{14}C]lactate (2 mM) or [U-^{14}C]pyruvate (2 mM) were provided as substrates, (*S*)-α-chlorohydrin (10 mM) had no inhibitory effect. (*R*)-α-Chlorohydrin

Table 1. Effect of increasing concentrations of (*S*)-α-chlorohydrin on the oxidation of D-[U-^{14}C]fructose (1 mM) to $^{14}CO_2$ by boar spermatozoa for 1 h at 34°C

Values, given as a percentage of duplicate controls, are the mean ±s.e.m. of four experiments

Concn of (*S*)-α-chlorohydrin in incubation medium (mM)	CO_2 produced (as % of control)	Concn of (*S*)-α-chlorohydrin in incubation medium (mM)	CO_2 produced (as % of control)
0·005	96·6±3·4	0·4	34·2±7·5
0·01	97·7±3·9	0·5	31·3±4·6
0·1	70·2±7·3	1·0	21·8±3·0
0·2	51·9±10·1	5·0	16·4±5·6
0·3	40·3±8·1	10·0	9·0±1·0

(100 mM) had no significant effect on the oxidation of any of the three substrates to CO_2. As the oxidative metabolism of lactate and pyruvate was unaffected, the site of the inhibitory action of (*S*)-α-chlorohydrin is at or beyond the entry of fructose into the glycolytic pathway and before the production of pyruvate.

Table 2. Glycolytic intermediates in boar spermatozoa after incubation with D-fructose (1 mM) at 34°C in the presence and absence of (*S*)-α-chlorohydrin

Values are the mean ±s.e.m. of four experiments; n.d., not detectable

Intermediate	Concn of intermediate (pmol/mg protein) in presence of (*S*)-α-chlorohydrin at concn (mM) of: 0	0·05	0·5
Fructose-1,6-bisphosphate	3·1±3·0	1690±37	5330±1160
Dihydroxyacetone phosphate	60·5±3·1	3630±660	4020±640
Glyceraldehyde 3-phosphate	n.d.	167±73	558±140
3-Phosphoglycerate	n.d.	n.d.	n.d.
2-Phosphoglycerate	n.d.	n.d.	n.d.
Pyruvate	316±92	n.d.	n.d.
Lactate	21900±7000	2910±1100	976±520

Effect on Glycolytic Intermediates and Adenine Nucleotides

Boar spermatozoa were incubated in the presence of (*S*)-α-chlorohydrin (0, 0·05 and 0·5 mM) with D-fructose (1 mM) as substrate. When key glycolytic intermediates were assayed, fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were shown to have accumulated (Table 2) indicating inhibition of the glycolytic pathway beyond the formation of the triose phosphates. The

depletion of pyruvate and lactate, together with the fact that there was no accumulation of either 2- or 3-phosphoglycerates, located the site of action of (*S*)-α-chlorohydrin as being an inhibition of glyceraldehydephosphate dehydrogenase.

When boar spermatozoa were incubated with D-fructose (1 mM) in the presence of (*S*)-α-chlorohydrin (0·05–0·5 mM), there was no effect on the adenine nucleotide pool but there was a decrease in the energy charge potential (Table 3). This is the first demonstration that the decrease in the energy charge potential of spermatozoa is due solely to the (*S*)-isomer of α-chlorohydrin since an equimolar mixture of the (*R*)- and the (*S*)-isomer (0·5 mM each), or of the racemic mixture (1 mM) had a similar effect as the (*S*)-isomer (0·5 mM) alone, whereas the (*R*)-isomer (100 mM) had no significant inhibitory activity.

Table 3. Effect of (*R*)-, (*S*)- and (*R,S*)-α-chlorohydrin on the concentrations of adenine nucleotides in boar spermatozoa at zero time and after incubation with D-fructose (1 mM) for 1 h at 34°C

Values are the mean ±s.e.m. of four experiments. ***Significantly different from control, $P < 0·001$

Isomer of α-chlorohydrin	Concn (mM)	Period of incubn (h)	Nucleotide concn (nmol/mg protein)			Total nucleotide concn (nmol/mg protein)	Energy charge potential
			ATP	ADP	AMP		
—	—	0	8·29±0·54	0·91±0·38	0·56±0·05	9·87±0·58	0·87±0·01
—	—	1[A]	7·78±0·55	3·31±0·41	0·88±0·17	11·7±0·99	0·80±0·02
(*S*)-	0·05	1	4·04±0·95	4·27±0·39	3·96±0·42	12·3±1·54	0·46±0·02***
(*S*)-	0·1	1	1·80±0·25	3·93±0·27	6·36±0·71	12·1±0·97	0·31±0·02***
(*S*)-	0·2	1	1·50±0·20	4·12±0·47	6·88±0·50	12·5±0·66	0·28±0·02***
(*S*)-	0·5	1	1·37±0·25	4·86±0·41	8·34±0·47	14·6±0·31	0·26±0·03***
(*R*)-+(*S*)-	0·5+0·5	1	1·73±0·38	4·22±0·49	7·24±0·45	12·6±0·66	0·28±0·01***
(*R,S*)-	1·0	1	1·49±0·28	3·27±0·23	7·53±0·61	11·3±1·20	0·28±0·03***
(*R*)-	100	1	6·36±0·17	3·69±1·01	1·54±0·34	11·1±1·02	0·73±0·04

[A] Control.

Onset of Inhibitory Activity

Inhibition of the oxidation of D-[U-^{14}C]fructose (1 mM) to $^{14}CO_2$ in boar spermatozoa by (*S*)-α-chlorohydrin (0·2 mM) was studied over a period of 3 h. While the inhibitory activity increased with increasing periods of incubation, there was no significant effect for the first 0·3 h (Fig. 1). This suggested that during this time, either (*S*)-α-chlorohydrin was being converted into an active metabolite or that uptake of the compound into spermatozoa was time-dependent. The latter possibility was excluded when uptake studies of (*R,S*)-[3-^{36}Cl]-α-chlorohydrin revealed that rapid equilibration of the compound between spermatozoa and the incubation solution had occurred by 0·5 min.

Metabolism of α-Chlorohydrin by Boar Spermatozoa

When boar spermatozoa were incubated for 1 h with D-fructose (1 mM) and (*R,S*)-[3-^{36}Cl]-α-chlorohydrin (100 μM), radiochromatogram scanning of t.l.c. plates of the supernatant solution revealed the presence of α-chlorohydrin but not of 3-chlorolactaldehyde. However, the supernatant solution derived from the spermatozoal pellet subjected to ultrasonic disruption contained both compounds in the ratio of approximately 5:1. When the incubation was repeated but in the presence of

lower concentrations of (*R,S*)-[3-^{36}Cl]-α-chlorohydrin (50, 25 and 10 μM), a similar pattern was seen though the area due to radioactive α-chlorohydrin never fell below that due to radioactive 3-chlorolactaldehyde. Furthermore, when the incubation was carried out for periods of less than 0·25 h (see Fig. 1), radioactive 3-chlorolactaldehyde could not be detected. In the event that these results were an indication of

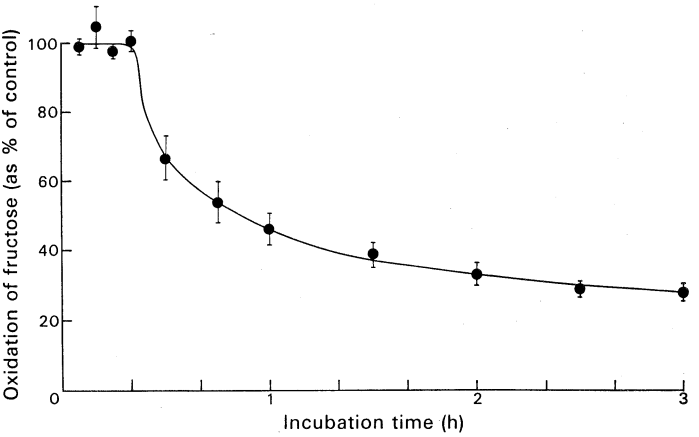

Fig. 1. Effect of (*S*)-α-chlorohydrin (0·2 mM) on the oxidation of D-[U-^{14}C]fructose (1 mM) to $^{14}CO_2$ by boar spermatozoa at 34°C. Values are the mean ±s.e.m. of five experiments.

the selective oxidative metabolism of one of the isomers of α-chlorohydrin, the metabolism of (*R*)-[3-^{36}Cl]-α-chlorohydrin by boar spermatozoa was investigated. With concentrations of this isomer from 100 μM to 1 mM, radioactive 3-chlorolactaldehyde could not be detected as a metabolite either in the supernatant derived from the incubation solution or that obtained from the spermatozoal pellet subjected to ultrasonic disruption, while (*R*)-[3-^{36}Cl]-α-chlorohydrin was present in both supernatant solutions.

Table 4. Glycolytic intermediates in boar spermatozoa after incubation with D-fructose (1 mM) for 1 h at 34°C in the presence and absence of (*R,S*)-3-chlorolactaldehyde

Values are the mean ±s.e.m. of four experiments; n.d., not detectable

Intermediate	Concn of intermediate (pmol/mg protein) in presence of (*R,S*)-3-chlorolactaldehyde at concn of:	
	0 mM	5 mM
Fructose-1,6-bisphosphate	13·4±10	2350±237
Dihydroxyacetone phosphate	61·9±30	1610±96
Glyceraldehyde 3-phosphate	n.d.	195±40

Effect of (R,S)*-3-Chlorolactaldehyde on the Oxidation of Fructose*

In the presence of (*R,S*)-3-chlorolactaldehyde (5 mM), the oxidation of D-[*U*-^{14}C]fructose (1 mM) to $^{14}CO_2$ by boar spermatozoa was inhibited by approximately 70% in 1 h. When key glycolytic intermediates were assayed, fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were shown

to have accumulated (Table 4) indicating that the site of inhibitory action was the reaction catalysed by glyceraldehydephosphate dehydrogenase.

The effect of (*R,S*)-3-chlorolactaldehyde (5 mM) on the oxidation of D-[U-^{14}C]-fructose to $^{14}CO_2$ was studied over a period of 90 min. Inhibitory activity was apparent within 2·5 min and had reached a maximum of approximately 30% of control values by 45 min (Fig. 2). This immediate inhibitory activity contrasts with that produced by (*S*)-α-chlorohydrin which is not evident until 20 min of incubation time.

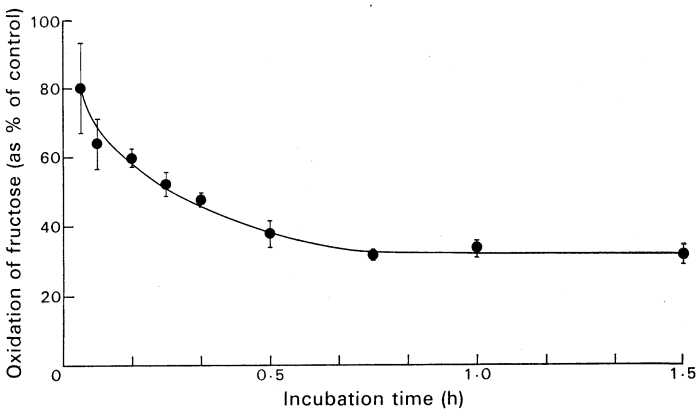

Fig. 2. Effect of (*R,S*)-3-chlorolactaldehyde (5 mM) on the oxidation of D-[U-^{14}C]fructose (1 mM) to $^{14}CO_2$ by boar spermatozoa at 34°C. Values are the mean ±s.e.m. of four experiments.

Discussion

Inhibition by (*R,S*)-α-chlorohydrin of the oxidation of fructose or glucose to CO_2 by rat, ram, boar, rhesus monkey and human spermatozoa *in vitro* has been well established (Hommonai *et al.* 1975; Brown-Woodman *et al.* 1978; Ford *et al.* 1979; Ford and Harrison 1980; Hutton *et al.* 1980). In the present study, this inhibitory effect on the metabolism of boar spermatozoa has been shown to be confined to the (*S*)-isomer of α-chlorohydrin. With the knowledge that (*S*)-α-chlorohydrin is the effective antifertility isomer when it is administered to male rats (Jackson *et al.* 1977), it appears that inhibition of fructolysis or glycolysis in the sperm of a number of species may be due specifically to this isomer.

The action of (*S*)-α-chlorohydrin in boar spermatozoa is an inhibition of the reaction involving glyceraldehydephosphate dehydrogenase which results in an increase in the amounts of the glycolytic intermediates fructose-1,6-bisphosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, and a depletion of those intermediates beyond the formation of 1,3-diphosphoglycerate, including lactate (Table 2). Similar effects have been observed when ram and rhesus monkey spermatozoa were incubated with (*R,S*)-α-chlorohydrin (Brown-Woodman *et al.* 1978; Ford and Harrison 1980) but the present study is the first to demonstrate that it is the (*S*)-isomer that is effective and that the (*R*)-isomer is devoid of any inhibitory activity. Furthermore, the degree of inhibition observed on ram and rhesus monkey spermatozoa was not as marked indicating that boar spermatozoa are

more susceptible to the inhibitory action of (*S*)-α-chlorohydrin than are those from the other species.

Inhibition of the reaction catalysed by glyceraldehydephosphate dehydrogenase in boar spermatozoa by (*S*)-α-chlorohydrin caused an increase in the concentration of AMP at the expense of ATP (Table 3). Although a number of respiratory substrates can be used by spermatozoa, those from many species (including the boar) exhibit a high rate of fructolysis and their energy demands can be fulfilled by substrate-level phosphorylation (Ford and Harrison 1981; Mann and Lutwak-Mann 1981). This is confirmed by the decrease in the energy charge potential of boar spermatozoa when they are incubated in the presence of (*S*)-α-chlorohydrin. For example, at a concentration of 0·5 mM, the (*S*)-isomer reduced the energy charge potential to 34% of control values in 1 h. By contrast, the energy charge potential of rhesus monkey spermatozoa was effected in a similar manner in the same period of time but only in the presence of 5 mM (*R,S*)-α-chlorohydrin (Ford and Harrison 1980), which is five times the effective concentration of the (*S*)-isomer on boar spermatozoa. It seems, therefore, that mature spermatozoa of those species susceptible to the action of α-chlorohydrin are rendered non-functional because they cannot utilize fructose, their major source of energy for the production of ATP (Brown-Woodman *et al.* 1975). When they are ejaculated, affected sperm would be morphologically normal but have such diminished motility that fertilization could not be successful.

The inhibitory action of (*R,S*)-α-chlorohydrin on the metabolic activity of ram spermatozoa has been shown not to occur immediately but only after a period of incubation (Mohri *et al.* 1975). This observation led to the suggestion that the true inhibitory compound was a metabolite that was being produced *in situ*. It was further suggested, with no substantiating evidence, that this metabolite was α-chlorohydrin-1-phosphate (3-chloropropan-1,2-diol-1-phosphate), formed by reaction of α-chlorohydrin with glycerol kinase (EC 2.7.1.30) (Mohri *et al.* 1975). Although an early report stated that α-chlorohydrin was a substrate for glycerol kinase (Thorner 1972), subsequent studies using purified, glycerol-free (*R,S*)-α-chlorohydrin showed that this was incorrect (Brooks 1979; Jones *et al.* 1981). Investigations into the metabolism of (*R,S*)-[3-^{36}Cl]-α-chlorohydrin by boar spermatozoa did not reveal the formation of α-chlorohydrin-1-phosphate but did show that the compound underwent oxidation to 3-chlorolactaldehyde of unspecified configuration (Jones *et al.* 1981).

We have confirmed that, as with the effect of (*R,S*)-α-chlorohydrin on ram spermatozoa (Mohri *et al.* 1975), there is a delay in the onset of inhibitory activity of (*S*)-α-chlorohydrin on boar spermatozoa (Fig. 1). When boar spermatozoa were incubated in the presence of (*R,S*)-[3-^{36}Cl]-α-chlorohydrin, uptake studies revealed that there was rapid equilibration of radioactive α-chlorohydrin between the incubation solution and the spermatozoa so that this delay was not due to a time-dependent process of cellular transport. By comparing the metabolism of (*R*)-[3-^{36}Cl]-α-chlorohydrin and (*R,S*)-[3-^{36}Cl]-α-chlorohydrin by boar spermatozoa, we have been able to deduce that it is the (*S*)-isomer that is undergoing oxidation to (*R*)-3-chlorolactaldehyde.* This (*R*)-aldehyde has the same absolute configuration as D- (or *R*-) glyceraldehyde

* When the (*R,S*)-nomenclature is applied to the chiral carbon of α-chlorohydrin, oxidation of $-CH_2OH$ to $-CHO$ results in a change in sequence of the substituent groups. Thus (*S*)-α-chlorohydrin is oxidized to (*R*)-3-chlorolactaldehyde.

and, consequently, as D- (or *R*-) glyceraldehyde 3-phosphate, the substrate for glyceraldehydephosphate dehydrogenase (Fig. 3).

There are, therefore, two stereochemical specificities involved in the inhibition of fructolysis in boar spermatozoa by α-chlorohydrin. Firstly, it is the (*S*)-isomer of α-chlorohydrin that is specific in affecting the reaction catalysed by glyceraldehydephosphate dehydrogenase and secondly, it is this isomer that is specifically oxidized to an aldehyde of identical configuration to that of the substrate for this enzyme. It would appear that (*R*)-3-chlorolactaldehyde could be the active metabolite since mechanistic considerations favour the presence of an aldehyde group in an inhibitor of glyceraldehydephosphate dehydrogenase. Active site attachment to the enzyme involves the formation of a thiohemiacetal link between the thiol group of a cysteine residue (Cys_{149}) and the aldehyde group of its substrate (Walsh 1979).

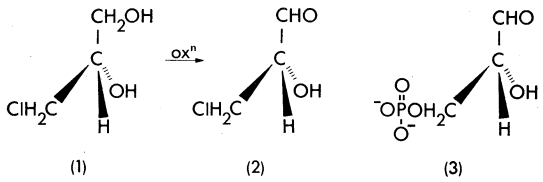

Fig. 3. Absolute stereochemistry of (*S*)-α-chlorohydrin (1), (*R*)-3-chlorolactaldehyde (2) and (*R*)-glyceraldehyde 3-phosphate (3).

Although (*R*)-3-chlorolactaldehyde is unavailable, a simple synthesis of the racemic mixture has been reported (Williams *et al.* 1960). When (*R,S*)-3-chlorolactaldehyde was added to boar spermatozoa which were incubated in the presence of D-[U-^{14}C]-fructose, there was an immediate inhibition of the production of $^{14}CO_2$ (Fig. 2). Analysis of key glycolytic intermediates revealed that there was an accumulation of fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Table 4) analogous to the effect produced by (*S*)-α-chlorohydrin. However, this similar response is only observed with 5 mM (*R,S*)-3-chlorolactaldehyde compared to a concentration of 0·2 mM (*S*)-α-chlorohydrin. While this concentration of exogenous (*R*)-3-chlorolactaldehyde is 10–12 times that of the (*S*)-α-chlorohydrin, it may be argued that if this is the inhibitory metabolite, there could be a barrier to it gaining access to the spermatozoal cytoplasm from the incubation solution. While this is conjecture at this stage, there is some evidence to suggest that this might be so. When (*R,S*)-[3-^{36}Cl]-α-chlorohydrin is incubated with boar spermatozoa, (*R*)-[3-^{36}Cl]-chlorolactaldehyde cannot be detected in the supernatant solution but is found only in the supernatant solution derived from the spermatozoa after they have been disrupted by sonic oscillation. This may be evidence that passage of (*R*)-3-chlorolactaldehyde across the spermatozoal membrane is not as facile as is the case with both isomers of α-chlorohydrin.

The antifertility action of α-chlorohydrin *in vitro* is species-specific. While affecting male fertility in a number of species, there is no activity in either the rabbit or the mouse (Ericsson 1970; Back *et al.* 1975). If an antifertility response requires the formation of (*R*)-3-chlorolactaldehyde within mature spermatozoa, it may be that the spermatozoa of these two species are incapable of carrying out the necessary biotransformation. While the metabolism of α-chlorohydrin in mouse spermatozoa has not been studied, rabbit spermatozoa are known not to metabolize α-chlorohydrin

to the aldehyde (Jones *et al.* 1981). It may be, therefore, that the antiglycolytic activity of (*S*)-α-chlorohydrin is confined to those cells possessing the enzyme that converts the isomer into (*R*)-3-chlorolactaldehyde, and this would explain why (*S*)-α-chlorohydrin is not a general inhibitor of all glycolytic cells. The enzyme from boar spermatozoa is at present being characterized.

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