Inhibitory effects of (*S*)-3-chlorolactaldehyde on the metabolic activity of boar spermatozoa *in vitro**

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Summary. (*S*)- α -Chlorohydrin and 3-chloro-1-hydroxyacetone inhibited the oxidative metabolism of fructose by boar spermatozoa only after a period of incubation in which they presumably underwent conversion to (*S*)-3-chlorolactaldehyde, an inhibitor of sperm glyceraldehyde 3-phosphate dehydrogenase. With glycerol as substrate, 3-chloro-1-hydroxyacetone had a similar effect, (*S*)- α -chlorohydrin was ineffective while (*R,S*)-3-chlorolactaldehyde was immediately effective with either substrate. All three compounds caused the accumulation of fructose 1,6-bisphosphate and dihydroxyacetone phosphate from fructose but not from glycerol which led to the conclusion that inhibition of triosephosphate isomerase was also associated with the anti-glycolytic action of (*S*)-3-chlorolactaldehyde. (*S*)-3-Chlorolactaldehyde caused the depletion of ATP in incubates of boar spermatozoa metabolizing fructose but not glycerol. This suggests that futile substrate cycling may play an important function in the anti-glycolytic action of (*S*)-3-chlorolactaldehyde and/or that boar spermatozoa can synthesize ATP from glycerol by a mechanism not involving the glycolytic pathway.

Keywords: (*S*)- α -chlorohydrin; (*S*)-3-chlorolactaldehyde; 3-chloro-1-hydroxyacetone; metabolic inhibition; spermatozoa (boar)

Introduction

The effects of the male antifertility agent α -chlorohydrin (3-chloropropane-1,2-diol) have been extensively studied in many species *in vivo* and with spermatozoa *in vitro* (see Jones, 1978, 1983). Early biochemical studies involving mature spermatozoa obtained from the ram (Brown-Woodman *et al.*, 1978), rhesus monkey, rat, hamster and man (Ford *et al.*, 1979) and boar (Hutton *et al.*, 1980) pin-pointed the site of action as inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). With the realization that α -chlorohydrin was a racemic mixture of (*R*)- and (*S*)-isomers, and the subsequent synthesis of these separate isomers (see Porter & Jones, 1982), the antifertility activity *in vivo* and the inhibitory activity *in vitro* was demonstrated to be due to (*S*)- α -chlorohydrin (*I*, Fig. 1) (see Jones, 1983). The mechanism of action of this isomer was shown to be a consequence of its conversion *in situ* into (*S*)-3-chlorolactaldehyde (*II*) (Jones *et al.*, 1983). The action of this metabolite, the actual inhibitor of sperm glyceraldehyde 3-phosphate dehydrogenase, causes a decrease in the glycolytic flux, thereby limiting the ability of the affected spermatozoa to synthesize the ATP which is essential for motility once they have been ejaculated.

One of the problems associated with studies of the action of (*S*)- α -chlorohydrin has been the inability to achieve a chemical synthesis of (*S*)-3-chlorolactaldehyde. While the racemic mixture can be prepared (Williams *et al.*, 1960), its use in biochemical studies is hampered by its relative impermeability to the sperm membrane (Stevenson & Jones, 1982) and by complications arising from the presence of the contaminating (*R*)-isomer (Stevenson & Jones, 1984). Circumvention of this problem has been achieved with 3-chloro-1-hydroxyacetone (*III*) which is apparently metabolized by triosephosphate isomerase (EC 5.3.1.1) specifically to (*S*)-3-chlorolactaldehyde in boar (Jones *et al.*, 1986) and ram (Reid *et al.*, 1986) spermatozoa.

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Fig. 1. The structures of (*S*)- α -chlorohydrin (*I*), (*S*)-3-chlorolactaldehyde (*II*) and 3-chloro-1-hydroxyacetone (*III*).

In this paper we report the results of a comparative study of the actions of (*S*)- α -chlorohydrin, (*R,S*)-3-chlorolactaldehyde and 3-chloro-1-hydroxyacetone on the metabolic activity of boar spermatozoa.

Materials and Methods

Chemicals and substrates. (*S*)- α -Chlorohydrin (Porter & Jones, 1982), 3-chloro-1-hydroxyacetone (Jones *et al.*, 1986) and (*R,S*)-3-chlorolactaldehyde (Williams *et al.*, 1960) were synthesized or prepared to confirmed purity by established procedures. D-[U- 14 C]Fructose, D-[2- 14 C]glucose, D-[6- 14 C]glucose and [U- 14 C]glycerol were purchased from Amersham International plc (Amersham, HP7 9LL, U.K.). All enzymes, substrates and co-factors were obtained from Sigma Chemical Co. (St Louis, MO 63178, U.S.A.). All other chemicals and reagents were of analytical grade and all solutions were prepared in water purified by reverse osmosis.

Metabolic studies. For each experiment, epididymides were obtained from 20–40 mature boars (*Sus scrofa*) within 1 h of slaughter at the abattoir. Spermatozoa were flushed from the cauda epididymidis with a modified phosphate-buffered saline (PBS) (Stevenson & Jones, 1982), centrifuged (2000 *g* for 10 min) and resuspended in PBS. This procedure was repeated twice to lower the content of endogenous substrates and suspensions were finally prepared at 34°C at a concentration of 2–3 g/ml (20–35 mg protein/ml or $\sim 10^8$ cells/ml). Only those samples observed by microscopic examination to contain >90% motile spermatozoa were used in the metabolic studies. Incubations were performed in stoppered Warburg flasks shaken at 120 cycles/min at 34°C for various times, each incubate containing washed sperm suspension (0.9 ml), radioactive (50 nCi) or non-radioactive substrate (0.05 ml) and PBS, (*S*)- α -chlorohydrin, (*R,S*)-3-chlorolactaldehyde or 3-chloro-1-hydroxyacetone (0.05 ml) at the concentrations indicated in the text. Preincubation of the incubates was performed in a similar manner except that the radioactive substrate was added after 10 min (addition at zero time). Metabolically-derived $^{14}\text{CO}_2$ was collected (Dawson, 1977) and assayed in a Beckman model LB3800 liquid scintillation counter by standard procedures (Stevenson & Jones, 1982). Neutralized and deproteinized incubation solutions (Stevenson & Jones, 1982) were used for the assays of fructose 1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Michal & Beutler, 1975) and ATP (Lamprecht & Trautschold, 1963). Protein was estimated (Lowry *et al.*, 1951), using bovine serum albumin as a standard, and the results were calculated using the linear transform equation of Coakley & James (1978).

Assays of enzyme activities. Suspensions (approx. 10%) of washed boar spermatozoa in PBS (20 ml) were incubated in unstoppered 50 ml conical flasks shaken at 120 cycles/min at 34°C. The substrate was D-fructose (1 mM) and the incubates contained (*R,S*)-3-chlorolactaldehyde (5 mM) or 3-chloro-1-hydroxyacetone (0.5 mM). After 0.5, 10 and 60 min, samples (5 ml), together with those from appropriate control incubations, were removed, placed immediately on ice then centrifuged at 300 *g* for 5 min. The sperm pellets were suspended in sucrose (0.25 M, 1.25 ml) containing EDTA (1 mM) and mercaptoethanol (1 mM), and disrupted by the addition of Triton X-100 (0.14 ml, 1%). The suspensions were centrifuged at 20 000 *g* for 10 min at 4°C and the supernatant solutions were used for the assays of enzyme activity. Each incubate was assayed for protein and the activity of one of the enzymes, glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase or fructose-bisphosphate aldolase (EC 4.1.2.13) (Bergmeyer *et al.*, 1974) in a Varian A 634 spectrophotometer.

Results

Inhibition of the oxidation of fructose to CO₂

The effects of (*S*)- α -chlorohydrin, 3-chloro-1-hydroxyacetone and (*R,S*)-3-chlorolactaldehyde on the oxidation of D-[U- 14 C]fructose to $^{14}\text{CO}_2$ by boar spermatozoa are shown in Fig. 2. The inhibitory actions of (*S*)- α -chlorohydrin and 3-chloro-1-hydroxyacetone were not immediate when they were added at zero time but only after incubation periods of 5 and 10 min, respectively.

Fig. 2. The effect of (a) (S)- α -chlorohydrin (0.5 mM), (b) 3-chloro-1-hydroxyacetone (0.5 mM) and (c) (R,S)-3-chlorolactaldehyde (5 mM) on the production of $^{14}\text{CO}_2$ from D-[U- ^{14}C]fructose (1 mM) by boar spermatozoa at 34°C. . . . , Preincubation for 10 min before addition of fructose. Panels (d), (e) and (f) represent the amounts of dihydroxyacetone phosphate (—●—), glycer-3-phosphate (—■—) and fructose 1,6-bisphosphate (—▲—) in the respective preincubates. Values are mean $\pm$ s.e.m. ($n = 3$).

Preincubation of the spermatozoa with these compounds for 10 min before addition of the substrate abolished the delay in inhibitory activity to give an immediate response similar to that observed with (R,S)-3-chlorolactaldehyde. Accompanying each of the zero-time incubations was the concomitant accumulation of dihydroxyacetone phosphate and substantial quantities of fructose 1,6-bisphosphate.

Inhibition of the oxidation of glycerol to CO_2

The effects of (S)- α -chlorohydrin, 3-chloro-1-hydroxyacetone and (R,S)-3-chlorolactaldehyde on the oxidation of [U- ^{14}C]glycerol to $^{14}\text{CO}_2$ by boar spermatozoa are shown in Fig. 3. When added at zero time, (S)- α -chlorohydrin had no inhibitory activity, 3-chloro-1-hydroxyacetone had a delayed effect similar to that when D-[U- ^{14}C]fructose was the substrate, whereas (R,S)-3-chlorolactaldehyde caused an immediate inhibitory response. Preincubation of the spermatozoa with (S)- α -chlorohydrin or 3-chloro-1-hydroxyacetone for 10 min before addition of the substrate resulted in immediate inhibitory actions by both compounds. During the course of the zero-time incubations there was no accumulation of dihydroxyacetone phosphate or fructose 1,6-bisphosphate.

Inhibition of the oxidation of glucose to CO_2

The effects of 3-chloro-1-hydroxyacetone and (R,S)-3-chlorolactaldehyde on the oxidation of D-[2- ^{14}C]glucose and D-[6- ^{14}C]glucose to $^{14}\text{CO}_2$ by boar spermatozoa are shown in Table 1. There

Fig. 3. The effect of (a) (*S*)- α -chlorohydrin (0.5 mM), (b) 3-chloro-1-hydroxyacetone (0.5 mM) and (c) (*R,S*)-3-chlorolactaldehyde (5 mM) on the production of ¹⁴CO₂ from [U-¹⁴C]glycerol (2 mM) by boar spermatozoa at 34°C. . . . , Preincubation for 10 min before addition of glycerol. Panels (d), (e) and (f) represent the amounts of dihydroxyacetone phosphate (—●—), glyceraldehyde 3-phosphate (—■—) and fructose 1,6-bisphosphate (—▲—) in the respective pre-incubates. Values are mean $\pm$ s.e.m. ($n = 3$). Note that the abscissa scale on the lower panels is different from that of Fig. 2.

Table 1. The effect of 3-chloro-1-hydroxyacetone (0.5 and 1 mM) and (*R,S*)-3-chlorolactaldehyde (5 mM) on the oxidation of D-[2-¹⁴C]glucose (1 mM) and D-[6-¹⁴C]glucose (1 mM) to ¹⁴CO₂ by washed boar spermatozoa incubated for 1 h at 34°C

Substrate	3-Chloro-1-hydroxyacetone		(<i>R,S</i>)-3-Chlorolactaldehyde 5 mM
	0.5 mM	1 mM	
D-[2- ¹⁴ C]Glucose	38.5 $\pm$ 6.3	6.6 $\pm$ 2.2*	41.8 $\pm$ 7.3
D-[6- ¹⁴ C]Glucose	38.8 $\pm$ 10.1	14.3 $\pm$ 1.0	34.8 $\pm$ 5.2

Values are mean $\pm$ s.e.m. ($n = 4$) expressed as % of control values.
* $P < 0.05$ compared with value for D-[6-¹⁴C]glucose (determined by one-way analysis of variance and multiple comparisons with controls by the Newman-Keuls method: Winer, 1971).

Table 2. The activities of four enzymes in boar spermatozoa after incubation at 34°C with fructose (1 mM) and 3-chloro-1-hydroxyacetone (0.5 mM) or (R,S)-3-chlorolactaldehyde (5 mM)

Inhibitor	Time (min)	Enzyme activity*		
		% of control	Control value	n
<i>Glycerol 3-phosphate dehydrogenase</i>				
3-Chloro-1-hydroxyacetone	0.5	122.5 ± 16.0	5.1 ± 0.7	5
	10	118.0 ± 9.4	6.7 ± 0.7	5
	60	102.0 ± 9.0	4.2 ± 0.6	4
(R,S)-3-Chlorolactaldehyde	0.5	118.9 ± 2.5	5.2 ± 0.4	4
	10	123.0 ± 11.7	4.6 ± 0.7	3
	60	180.6 ± 13.2	3.7 ± 0.1	4
<i>Glyceraldehyde 3-phosphate dehydrogenase</i>				
3-Chloro-1-hydroxyacetone	0.5	65.4 ± 2.9	87.0 ± 8.7	3
	10	41.5 ± 1.2	103.0 ± 10.2	4
	60	28.6 ± 2.9	122.0 ± 11.8	4
(R,S)-3-Chlorolactaldehyde	0.5	99.3 ± 6.3	70.3 ± 8.3	4
	10	88.6 ± 7.7	83.5 ± 11.2	4
	60	63.9 ± 4.8	115.4 ± 14.4	4
<i>Fructose-bisphosphate aldolase</i>				
3-Chloro-1-hydroxyacetone	0.5	120.7 ± 3.0	24.9 ± 3.0	5
	10	139.9 ± 6.2	23.0 ± 2.8	6
	60	152.9 ± 10.3	20.5 ± 3.3	4
(R,S)-3-Chlorolactaldehyde	0.5	114.1 ± 12.9	13.9 ± 1.5	6
	10	76.6 ± 7.0	20.4 ± 2.0	5
	60	110.4 ± 10.9	17.5 ± 1.8	6
<i>Triosephosphate isomerase</i>				
3-Chloro-1-hydroxyacetone	0.5	79.3 ± 4.3	1.16 ± 0.16	5
	10	43.0 ± 1.6	1.43 ± 0.09	5
	60	31.9 ± 4.0	1.40 ± 0.06	5
(R,S)-3-Chlorolactaldehyde	0.5	105.8 ± 2.3	1.25 ± 0.04	4
	10	102.2 ± 5.5	1.32 ± 0.05	4
	60	29.2 ± 5.2	1.40 ± 0.08	4

*Values (mean ± s.e.m. for the number (n) of determinations) are expressed as nmol substrate converted/min/mg protein except for triosephosphate isomerase which is μmol substrate converted/min/mg protein.

was no significant difference in the inhibitory activity of (R,S)-3-chlorolactaldehyde (5 mM) or 3-chloro-1-hydroxyacetone (0.5 mM) on the amount of $^{14}\text{CO}_2$ produced from either radioactive substrate, but at a concentration of 1 mM, 3-chloro-1-hydroxyacetone exhibited a greater effect on the production of $^{14}\text{CO}_2$ from [2- ^{14}C]glucose than from [6- ^{14}C]glucose.

Effect of 3-chloro-1-hydroxyacetone and (R,S)-3-chlorolactaldehyde on enzyme activity

When boar spermatozoa were incubated for 1 h at 34°C with D-fructose (1 mM) as the substrate in the presence and absence of 3-chloro-1-hydroxyacetone (0.5 mM) or (R,S)-3-chlorolactaldehyde (5 mM), neither compound affected the activity of glycerol 3-phosphate dehydrogenase or fructose-bisphosphate aldolase but both had a time-dependent inhibitory action on the activity of glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase (Table 2).

Inhibition of the synthesis of ATP

The effects of (S)-α-chlorohydrin (0.5 mM), 3-chloro-1-hydroxyacetone (0.5 mM) and (R,S)-3-chlorolactaldehyde (5 mM) on the concentration of ATP in washed boar spermatozoa incubated for

Table 3. The concentration of ATP in washed boar spermatozoa incubated for 1 h at 34°C with either fructose (1 mM) or glycerol (2 mM) as substrates in the presence and absence of (*S*)- α -chlorohydrin (0.5 mM), 3-chloro-1-hydroxyacetone (0.5 mM) or (*R,S*)-3-chlorolactaldehyde (5 mM)

Inhibitor	Time (min)	ATP conc. (nmol/mg protein)	
		Fructose	Glycerol
—	0	2.05 $\pm$ 0.90	3.03 $\pm$ 0.74
—	60	9.26 $\pm$ 0.25	9.37 $\pm$ 1.48
(<i>S</i>)- α -Chlorohydrin	60	1.37 $\pm$ 0.25	9.58 $\pm$ 1.94
3-Chloro-1-hydroxyacetone	60	2.34 $\pm$ 0.67	5.75 $\pm$ 0.18
(<i>R,S</i>)-3-Chlorolactaldehyde	60	2.70 $\pm$ 0.42	8.32 $\pm$ 0.21

Values are mean $\pm$ s.e.m. ($n = 4$).

1 h at 34°C are shown in Table 3. With fructose (1 mM) as the substrate, the presence of all three compounds inhibited the synthesis of ATP; however, with glycerol (2 mM) as the substrate, (*S*)- α -chlorohydrin did not inhibit ATP synthesis whereas 3-chloro-1-hydroxyacetone and (*R,S*)-3-chlorolactaldehyde only partly affected its production compared to control incubations.

Discussion

In the presence of (*S*)- α -chlorohydrin, the oxidative metabolism of fructose by boar spermatozoa was inhibited since these cells possess an NADP⁺-dependent dehydrogenase which is capable of metabolizing (*S*)- α -chlorohydrin to (*S*)-3-chlorolactaldehyde (Stevenson & Jones, 1985). The formation of this metabolite, which is an inhibitor of glyceraldehyde 3-phosphate dehydrogenase, is reflected by a delay (of ~ 5 min with 0.5 mM-(*S*)- α -chlorohydrin) before the production of CO₂ is affected, a delay which was eliminated when the spermatozoa were preincubated with (*S*)- α -chlorohydrin for 10 min before the fructose was added. A similar situation was seen when boar spermatozoa were incubated with 3-chloro-1-hydroxyacetone, which is also metabolized to (*S*)-3-chlorolactaldehyde; a delay in the inhibitory effect (of ~ 10 min at a concentration of 0.5 mM) which was not apparent with a 10-min preincubation period. When (*R,S*)-3-chlorolactaldehyde was added to boar spermatozoa metabolizing fructose, the inhibitory action was immediate. Accompanying the inhibitory actions of these three compounds was the accumulation of fructose 1,6-bisphosphate and dihydroxyacetone phosphate which is indicative of a block in the glycolytic pathway involving glyceraldehyde 3-phosphate dehydrogenase. Moreover, all three compounds prevented the attainment of ATP concentrations comparable to that of control incubations. While inhibition of glycolysis at the step catalysed by glyceraldehyde 3-phosphate dehydrogenase would lower the ability of the cells to generate ATP by both oxidative- and substrate-level phosphorylation, it has been proposed by Ford & Harrison (1985, 1987) that the accumulation of fructose 1,6-bisphosphate contributes to ATP dissipation by initiating futile substrate cycling.

A different situation emerged when the oxidative metabolism of glycerol by boar spermatozoa was studied in the presence of each of the three compounds. The ineffectiveness of (*S*)- α -chlorohydrin unless preincubated for 10 min is probably due to competition between glycerol and (*S*)- α -chlorohydrin for the NADP⁺-dependent dehydrogenase so that insufficient (*S*)-3-chlorolactaldehyde was produced (Stevenson & Jones, 1985). The presence of 3-chloro-1-hydroxyacetone resulted in a delay in the onset of inhibition (abolished by preincubation) whereas the effect of (*R,S*)-3-chlorolactaldehyde was immediate. However, the inhibitory actions of 3-chloro-1-hydroxyacetone and (*R,S*)-3-chlorolactaldehyde were not accompanied by the accumulation of fructose 1,6-bisphosphate or dihydroxyacetone phosphate, the amounts of which

Fig. 4. The sites of inhibitory action of (*S*)-3-chlorolactaldehyde (II) on the glycolytic pathway in boar spermatozoa.

remained low and comparable to those produced in the presence of (*S*)- α -chlorohydrin which caused no inhibition unless preincubated (Fig. 3). The failure of fructose 1,6-bisphosphate to accumulate when 3-chloro-1-hydroxyacetone or (*R,S*)-3-chlorolactaldehyde were present could be rationalized on the basis that triosephosphate isomerase was being inhibited. Such an action would limit the formation of sufficient glyceraldehyde 3-phosphate necessary for the production of fructose 1,6-bisphosphate by fructose-bisphosphate aldolase (Fig. 4). Further evidence in support of this proposal was derived from an examination of the relative degrees of inhibition of $^{14}\text{CO}_2$ produced from specifically-labelled [^{14}C]glucose in the presence of 1.0 mM-3-chloro-1-hydroxyacetone (Table 1). The greater degree of effect upon [$2\text{-}^{14}\text{C}$]glucose, compared to that upon [$6\text{-}^{14}\text{C}$]glucose, is in accordance with the inhibition of triosephosphate isomerase. This was substantiated by an examination of the effects of both inhibitors on several enzymes associated with this region of the glycolytic pathway. When incubated with boar spermatozoa, neither compound impaired the activities of glyceraldehyde 3-phosphate dehydrogenase or fructose-bisphosphate aldolase but both inhibited the activities of glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase in a time-dependent manner.

While this is the first demonstration that the anti-glycolytic action of (*S*)-3-chlorolactaldehyde involves the inhibition of two glycolytic enzymes in [boar] spermatozoa, the susceptibility of sperm triosephosphate isomerase to inhibition has been mentioned, if not recognized, on three previous occasions. Brown-Woodman *et al.* (1978) showed that when washed ram spermatozoa were incubated with (*R,S*)- α -chlorohydrin, the activities of glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase and fructose-bisphosphate aldolase were depressed to 7, 21 and 62% of control values, respectively. Even though the conditions used were more severe (3 mM-(*R,S*)- α -chlorohydrin, $\equiv$ 1.5 mM-(*S*)- α -chlorohydrin, for 3 h) than those in the present study, the susceptibility of these three enzymes to inhibition is, in retrospect, not surprising since they all accept (*R*)-glyceraldehyde 3-phosphate as a substrate and (*S*)-3-chlorolactaldehyde has a similar structure to, and the identical stereochemistry of, this substrate. The second mention was in a study involving 6-chloro-6-deoxyglucose which has an anti-glycolytic action on mature spermatozoa dependent on its metabolism to (*S*)-3-chlorolactaldehyde (Ford, 1982; Jones & Ford, 1984). After oral administration of the chloro-sugar to rats for 14 days, the activities of several glycolytic enzymes in caudal spermatozoa were assessed at the end of the treatment period (Ford & Waites, 1981; Ford *et al.*, 1981). Animals receiving 12 mg/kg/day contained spermatozoa with glyceraldehyde 3-phosphate dehydrogenase activity 48% that of controls and triosephosphate isomerase activity 59% that of controls while those receiving 24 mg/kg/day contained spermatozoa with activities of 19% and 28% of control values, respectively. Neither treatment regimen affected the activity of fructose-

bisphosphate aldolase. The third mention concerns "the extensive retention of ^3H from $[3\text{-}^3\text{H}]\text{glucose}$ in fructose 1,6-bisphosphate in boar spermatozoa in the presence of $[R,S]\text{-}\alpha\text{-chlorohydrin}$ " (Ford & Harrison, 1987). The isomerization reaction catalysed by triosephosphate isomerase would liberate ^3H originating from $[3\text{-}^3\text{H}]\text{glucose}$ so that its retention is indicative of the enzyme being inhibited.

An interesting outcome of the present study is that 3-chloro-1-hydroxyacetone and $(R,S)\text{-3-chlorolactaldehyde}$ do not depress the production of ATP by boar spermatozoa metabolizing glycerol to the same extent as when the substrate is fructose. Inhibition of glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase would decrease the glycolytic flux to the same extent whether the substrate was fructose or glycerol and this is demonstrated by a similar reduction in the production of $^{14}\text{CO}_2$ from either ^{14}C -labelled substrate with either inhibitor. A possible explanation for this is that boar spermatozoa have the ability to generate ATP from glycerol (or glycerol 3-phosphate) by a mechanism which does not involve recourse to stage two of the glycolytic pathway and this is currently being investigated. For example, the production in 1 h of equivalent amounts of ATP (Table 2) required the liberation of almost twice the amount of $^{14}\text{CO}_2$ from D-[U- ^{14}C]fructose (1 mM) than from [U- ^{14}C]glycerol (2 mM) (A. R. Jones, unpublished). Such an ability has been recognized in guinea-pig spermatozoa (Frenkel *et al.*, 1975) and White (1957) has observed that "fructolysis by bull spermatozoa is increased under anaerobic conditions [but] the breakdown of glycerol was significantly reduced".

The fact that when glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase are inhibited ATP dissipation in boar spermatozoa is not as pronounced when glycerol is the substrate as it is when glucose or fructose are metabolized may support the proposal that futile substrate cycling is a contributory factor in the anti-glycolytic action of $(S)\text{-}\alpha\text{-chlorohydrin}$ (Ford & Harrison, 1987). These authors have proposed that the accumulation of fructose 1,6-bisphosphate can lead to its interconversion to fructose 6-phosphate (Fig. 4), its conversion into fructose *via* fructose 1-phosphate, the isomerization of fructose 6-phosphate to glucose 6-phosphate and the hydrolysis of glucose 6-phosphate to glucose. The establishment of particular substrate cycles between these various intermediates would lead to the loss of ATP. As this would depend on there being an initial increase in the concentration of fructose 1,6-bisphosphate, this could only occur if the substrate was a hexose when triosephosphate isomerase is inhibited. Their alternative suggestion, that the increase in concentration of glycolytic intermediates (Ford & Harrison, 1986) depletes the supply of inorganic phosphate for oxidative phosphorylation (Ford & Harrison, 1987), seems unlikely since experimental incubations were performed in phosphate-buffered medium. Whatever the explanation, the mechanism of the anti-glycolytic action of those compounds metabolized to $(S)\text{-3-chlorolactaldehyde}$ is complex and will require a clearer understanding of the biochemistry of mature spermatozoa before it can be solved satisfactorily.

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