Effects of 6-chloro-6-deoxysugars on glucose oxidation in rat spermatozoa

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Summary. 6-Chloro-6-deoxyfructose or 6-chloro-6-deoxyglucitol (>90 μmol/kg/day), 6-chloro-6-deoxyglucose or 6-chloro-6-deoxymannose (>120 μmol/kg/day) and 6-chloro-6-deoxygalactose (>300 μmol/kg/day) all had an antifertility action in the male rat when given by mouth. Spermatozoa from the infertile rats were unable to oxidize glucose. This effect was always produced by a lower dose than the antifertility effect and the threshold dose for the 2 effects varied in a parallel fashion between the different 6-chloro-6-deoxysugars. Glucose oxidation appeared to be inhibited at the triose phosphate isomerase or glyceraldehyde 3-phosphate dehydrogenase reaction. These effects of 6-chloro-6-deoxysugars are similar to those of α-chlorohydrin.

The activities of the glycolytic enzymes were greatly in excess of the observed flux through the pathway and high concentrations of glucose 6-phosphate and triose phosphate accumulated in rat spermatozoa incubated with 2 mM-D-glucose.

Introduction

Several 6-chloro-6-deoxysugars have an antifertility action in the male rat (Ford & Waites, 1978a, b, 1980; Heitfeld, McRae & Vickery, 1979; Ford, 1980) and 6-chloro-6-deoxyglucose is also effective in the male marmoset monkey (J. P. Hearn, unpublished data). Their effect is similar to that of α-chlorohydrin (Jones, 1978) and it is believed that they act by preventing the metabolism of glucose by spermatozoa from treated animals.

In the present study the doses required to make male rats infertile and those needed to inhibit sperm glucose oxidation have been compared for 5 different 6-chloro-6-deoxysugars and an attempt has been made to locate the site of action of the drug in the glycolytic pathway.

Materials and Methods

Chemicals and enzymes

6-Chloro-6-deoxyglucose, 6-chloro-6-deoxyfructose, 6-chloro-6-deoxymannose, 6-chloro-6-deoxyglucitol and 6-chloro-6-deoxygalactose were synthesized and generously donated by Dr E. B. Rathbone and Dr P. J. Simpson (Philip Lyle Memorial Research Laboratory, Tate and Lyle Ltd, Reading, RG6 2BX, U.K.). 6-Chloro-6-deoxyfructose and 6-chloro-6-deoxyglucitol were syrups but the other compounds had a distinct melting point. All were free from discoloration and inorganic chlorides. The pH of a 10% (w/v) solution was neutral and the compounds appeared >95% pure by gas–liquid (Sweeley, Bentley, Makita & Wells, 1963) or
thin-layer (chloroform: methanol, 2:1 v/v, or ethanol: ethyl acetate: water 5:45:1 by vol., on 0.25 mm Merck Silica gel G) chromatography. D-[U-14C]Glucose was obtained from the Radiochemical Centre, Amersham, HP7 9LL, U.K. Enzymes and biochemicals were purchased from Boehringer Corp., Lewes, BN7 1LG, U.K., or Sigma Chemical Co. Ltd, Poole, BH17 7NH, U.K. Inorganic compounds, solvents, liquid scintillation materials and D-glucose were from BDH Chemicals Ltd, Poole, BH12 4NN, U.K., or from Fisons Scientific Apparatus, Loughborough, LE11 0R9, U.K.

Rats

CD rats were obtained from Charles River U.K. Ltd, Margate, CT9 4LT, U.K., at least 4 days before the start of an experiment. They were kept in 12 h light: 12 h dark (with the light period beginning at 07:00 h) and were given food (Diet 86: Dixons, Ware, Herts, U.K.) and water ad libitum.

Fertility trials and glucose oxidation by spermatozoa

The 6-chloro-6-deoxysugars were dissolved in water at such concentrations that each group of rats received 1 ml/kg. Groups of 6 male rats (300–450 g body wt) were dosed daily by oral gavage for 14 days. Each male was paired with a virgin female (180–250 g body wt) for the final 7 days. For some females, vaginal smears were taken each day between 09:00 and 11:00 h and were examined for the presence of spermatozoa. After 10 days separation from the males, the females were killed with chloroform. Live embryos, resorbing embryos and the corpora lutea were counted.

On the day after the final dose the male rats were killed by cervical dislocation. A cut was made in the cauda epididymis and spermatozoa were flushed out with phosphate-buffered saline (PBS) (143 mM-NaCl; 2.7 mM-KCl; 8.1 mM-Na2HPO4; 1.5 mM-KH2PO4; 0.8 mM-CaCl2; 0.5 mM-MgCl2; 0.1 mM-EDTA (tetraborate salt); bovine serum albumin (Cohn Fraction V) 0.1% w/v; 90 µg penicillin G/ml; 120 µg streptomycin sulphate/ml) infused through a plastic cannula inserted in the distal region of the vas deferens. Spermatozoa from 2 rats in the same group which had been processed simultaneously were pooled and dispersed in about 3.5 ml PBS by gently sucking in and out of a wide-mouthed Pasteur pipette. Sperm suspension (1-6 ml: 82–180 x 10^6 spermatozoa) was added to two 10 ml conical flasks sealed with Suba-seal caps (Gallenkamp, London EC2, U.K.) and D-[U-14C]glucose (0.40 ml; 4 µmol, 2 µCi) was added to each flask. Perchloric acid (1 ml, 1 ml) was added to one flask immediately before the addition of glucose and to the other after it had been incubated with shaking for 30 min at 35°C. Air was the gas phase for all incubations. 14CO2 was collected from each flask by piercing the cap with 2 hypodermic needles through which a stream of air was drawn through the flask and into a series of 2 scintillation vials each containing hyamine hydroxide (1.0 ml; 0.2 M solution in toluene) dispersed on glass wool or on glass-fibre filter paper. The recovery of 14CO2 from added NaHCO3 by this procedure was 101 ± 2.5% mean ± s.e.m., n = 8). The acidified sperm suspension was homogenized (Ultra-Turrax blender, 15 sec full speed) and the homogenate was centrifuged (600 g, 10 min, 2–4°C). The supernatant was neutralized with 0.40 ml 2.3 M-K2CO3 which contained 0.7 M-2-(N-morpholino)ethane sulphonic acid. KClO4 was removed by centrifugation (600 gav, 10 min, 2–4°C).

Radioactivity was measured in a liquid scintillation spectrometer (Nuclear Enterprises, Edinburgh). The concentration of spermatozoa was measured with a haemocytometer and at least 2 samples of each suspension were counted.
Enzyme activities in rat spermatozoa

The contents of each cauda epididymidis of each rat were expelled by forcing air down a cannula inserted into the vas deferens, and mixed with an equal volume of aqueous glycerol (50% v/v). The slurry was sucked into a number of capillary tubes and stored under liquid nitrogen. To prepare material for assay the contents of a tube were thawed and mixed with 100–200 times their own volume of ice-cold 10 mM-potassium phosphate buffer, pH 7.4. This lysed the plasma membrane of the spermatozoa (Keyhani & Storey, 1973) and portions of this suspension could be used for enzyme assays without further treatment. Protein was measured by the method of Warburg & Christian as described by Dawson, Elliott, Elliott & Jones (1969). All assays were performed at 25°C in a total volume of 2.0 ml and the rate of reaction was observed by continuously recording the absorbance at 340 nm in a Gilford Model 240 spectrophotometer. The rates given in Table 3 were obtained by subtracting the rate before the addition of substrate from the subsequent rate. The rate of reaction was always directly proportional to the amount of spermatozoa added. Conditions used for each assay are given below (buffers were adjusted to the stated pH at room temperature (about 20°C) by the addition of HCl or NaOH as appropriate).

Hexokinase (EC 2.7.1.1): 50 mM-triethanolamine, pH 7.4; 10 mM-MgCl₂; 5 mM-EDTA; 7.5 mM-KCl; 10 mM-ATP; 0.59 mM-NADP⁺; and 0.74 units glucose 6-phosphate dehydrogenase (EC 1.1.1.47). The reaction was started by adding 5 mM-glucose.

Phosphofructokinase (EC 2.7.1.11): 50 mM-tris–HCl, pH 8.2; 5 mM-MgCl₂; 200 mM-KCl; 1 mM-ATP; 2 mM-AMP; 0.14 mM-NADH; 0.45 units aldolase (EC 4.1.2.13); 50 units triose phosphate isomerase (EC 5.3.1.1); and 5 units glycerol 3-phosphate dehydrogenase (EC 1.1.1.8). The reaction was started by adding 4 mM-fructose 6-phosphate.

Aldolase (EC 4.1.2.13): 50 mM-Tris–HCl, pH 8.2; 5 mM-MgCl₂; 200 mM-KCl; 0.14 mM-NADH; 50 units triose phosphate isomerase; and 5 units glycerol 3-phosphate dehydrogenase. The reaction was started by adding 4 mM-fructose 1,6-bisphosphate.

Triose phosphate isomerase (EC 5.3.1.1): 50 mM-Tris–HCl, pH 8.2; 5 mM-MgCl₂; 200 mM-KCl; 0.14 mM-NADH; and 5 units glycerol 3-phosphate dehydrogenase. The reaction was started by the addition of 1 mM-glyceraldehyde 3-phosphate.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12): 50 mM-triethanolamine, pH 7.4; 10 mM-MgCl₂; 75 mM-KCl; 4 mM-ATP; 0.14 mM-NADH; and 9 units phosphoglycerate kinase (EC 2.7.2.3). The reaction was started by the addition of 5 mM-D-glyceraldehyde 3-phosphate.

Pyruvate kinase (EC 2.7.1.40): 50 mM-triethanolamine, pH 7.4; 10 mM-MgCl₂; 75 mM-KCl; 0.25 mM-ADP; 0.14 mM-NADH; and 2.5 units lactate dehydrogenase (EC 1.1.1.27). The reaction was initiated by the addition of 0.27 mM-phosphoenol pyruvate (cyclohexylammonium salt).

Lactate dehydrogenase (EC 1.1.1.27): 50 mM-triethanolamine, pH 7.4; 10 mM-MgCl₂; 75 mM-KCl; and 0.14 mM-NADH. The reaction was started by the addition of 0.5 mM-sodium pyruvate.

Oxygen consumption by spermatozoa

Spermatozoa (25–41 × 10⁶) in a total volume of 1.0 ml PBS were placed in the reaction chamber of an oxygen electrode (Rank Bros, Bottisham, Cambridge, U.K.) maintained at 35°C, and the substrate was added when a constant or slowly decreasing response was attained. Two electrodes were available and to minimize possible variation due to the ageing of spermatozoa measurements were made as soon as possible after collection of the cells; the order was glycerol 3-phosphate before pyruvate plus malate (Electrode 1) and lactate before succinate (Electrode 2). Separate samples of spermatozoa from the same rat were used for each substrate. The electrodes were calibrated against air-saturated water (0.2 mM-O₂ at 35°C).
Groups of 6 rats were treated with 6-chloro-6-deoxyglucose (120 µmol/kg/day) or water (controls) for 14 days. On Day 15 the rats were killed and spermatozoa were flushed from the cauda epididymidis as described above except that the PBS buffer contained 2 mM-D-glucose. The spermatozoa from each rat (27–163 × 10^6) were dispersed in 2.5 ml of the buffer and incubated for 40 min at 35°C. Samples (0.7 ml) were removed after 0 and 40 min incubation and mixed immediately with 0.35 ml 1 M-perchloric acid. The incubations were begun 1–2 min after dilution of the spermatozoa. The acidified samples were neutralized as described above, the volumes of reagents being adjusted accordingly. The concentrations of glucose 6-phosphate, fructose 1,6-bisphosphate, triose phosphate (dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate), glyceral 3-phosphate, glycercate 2-phosphate, phosphoenol pyruvate and pyruvate were assayed enzymically in a fluorometer (Maitra & Estabrook, 1964).

Results

The fertility of male rats and the oxidation of glucose by their spermatozoa

The mean ± s.e.m. numbers of spermatozoa obtained from the cauda epididymidis of control rats and of rats which became infertile were similar (2.56 ± 0.14 × 10^8 (n = 15) and 2.42 ± 0.12 × 10^8 (n = 18) spermatozoa/2 rats respectively) and the spermatozoa from the infertile rats appeared normal when examined by light microscopy (× 400 magnification). The weights of the testes and of the accessory organs of the infertile rats remained in the control range and the histology of these tissues was normal (Ford & Waites, 1980; J. R. P. Cabral & W. C. L. Ford, unpublished data). Spermatozoa were found in vaginal smears taken from nearly all females paired with treated males. The proportion of pairings in which mating was not confirmed was similar in the control group.

The dose of 6-chloro-6-deoxysugar required to produce infertility depended on the structure of the hexose moiety (Table 1). No embryos were sired by male rats given 6-chloro-6-deoxyfructose, 6-Chloro-6-deoxyglucitol, 6-Chloro-6-deoxyhexose, 6-Chloro-6-deoxymannose, 6-Chloro-6-deoxygalactose.

Table 1. The effect of various doses of some 6-chloro-6-deoxysugars on the fertility of male rats and on sperm glucose oxidation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (µmol (mg)/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (control)</td>
</tr>
<tr>
<td>6-Chloro-6-deoxyfructose</td>
<td></td>
</tr>
<tr>
<td>Conception rate</td>
<td>95.7</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>14.9 ± 5.9</td>
</tr>
<tr>
<td>6-Chloro-6-deoxyglucitol</td>
<td></td>
</tr>
<tr>
<td>Conception rate</td>
<td>94.6</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td>6-Chloro-6-deoxyhexose</td>
<td></td>
</tr>
<tr>
<td>Conception rate</td>
<td>85.6</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>10.5 ± 1.4</td>
</tr>
<tr>
<td>6-Chloro-6-deoxymannose</td>
<td></td>
</tr>
<tr>
<td>Conception rate</td>
<td>94.7</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>16.2 ± 4.1</td>
</tr>
<tr>
<td>6-Chloro-6-deoxygalactose</td>
<td></td>
</tr>
<tr>
<td>Conception rate</td>
<td>89.5</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>14.3 ± 2.8</td>
</tr>
</tbody>
</table>

Conception rate = (total live embryos + total resorbing embryos)/total no. of corpora lutea) × 100. 6 females/group.
Glucose oxidation = nmol [U-14C]glucose converted to 14CO2/30 min/10^8 spermatozoa. Values are mean ± s.e.m. for 3 observations.
Significantly different from control value: * P < 0.05, ** P < 0.01 (t test).
6-chloro-6-deoxyglucose (300 µmol/kg/day) or 6-chloro-6-deoxymannose (300 µmol/kg/day) or 6-chloro-6-deoxygalactose (300 µmol/kg/day) (D. Snodin, personal communication). The lowest dose which rendered the males infertile was sufficient to decrease the capacity of their spermatozoa to oxidize glucose to CO₂ by at least 90% (Table 1) and sometimes (e.g. 120 µmol 6-chloro-6-deoxygalactose/kg/day) glucose oxidation was substantially decreased by a dose which had little effect on fertility. However, the threshold dose for either effect varied in a similar way with the different 6-chloro-6-deoxyhexoses examined.

There was no increase in the concentration of lactate in rat spermatozoa incubated under these conditions and glucose was the only radioactive compound detected by t.l.c. after the incubations of the media.

By contrast, even prolonged treatment of rats with 6-chloro-6-deoxyglucose (120 mol/kg/day) did not affect the ability of their spermatozoa to oxidize glycerol 3-phosphate, lactate, succinate or pyruvate plus malate (Table 2).

**Table 2.** The oxidation of various substrates by epididymal spermatozoa from rats treated with 6-chloro-6-deoxyglucose

<table>
<thead>
<tr>
<th>Dose (µmol/mg)/kg/day</th>
<th>10 mm-rac-Glycerol 3-phosphate</th>
<th>10 mm-DL-Lactate</th>
<th>5 mm-Pyruvate plus 1 mm-L-malate</th>
<th>5 mm-Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>126 ± 22 (3)</td>
<td>19 ± 2.5 (5)</td>
<td>13 ± 1.1 (4)</td>
<td>16 (15.4; 16.5) (2)</td>
</tr>
<tr>
<td>120 (24)</td>
<td>131 ± 19 (5)</td>
<td>23 ± 3.1 (5)</td>
<td>14 ± 2.8 (5)</td>
<td>14 ± 1.4 (4)</td>
</tr>
</tbody>
</table>

The uptake of oxygen in the absence of substrate was <1.5 nmol/10⁸ spermatozoa/min. Values are mean ± s.e.m. for the no. of rats indicated in parentheses.

**The activities of the glycolytic enzymes**

The specific activities of the glycolytic enzymes in rat spermatozoa are considerably higher than would be needed to explain the observed rates of glucose oxidation (Table 3). The activities of glyceraldehyde 3-phosphate dehydrogenase and of triose phosphate isomerase were significantly decreased in spermatozoa from rats treated with 6-chloro-6-deoxyglucose (60 µmol/kg/day) compared to controls. When the dose of 6-chloro-6-deoxyglucose was increased to 120 µmol/kg/day the inhibition of these two enzymes was more pronounced and hexokinase, pyruvate kinase and lactate dehydrogenase were inhibited by a small but significant amount.

**Table 3.** The specific activities of some glycolytic enzymes in epididymal spermatozoa from rats given 6-chloro-6-deoxyglucose

<table>
<thead>
<tr>
<th>Dose (µmol (mg)/kg/day)</th>
<th>Hexokinase</th>
<th>Phosphofructokinase</th>
<th>Aldolase</th>
<th>Triose phosphate isomerase</th>
<th>Glyceraldehyde 3-phosphate dehydrogenase</th>
<th>Pyruvate kinase</th>
<th>Lactate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>570 ± 44</td>
<td>240 ± 37</td>
<td>390 ± 42</td>
<td>7220 ± 439</td>
<td>1020 ± 151</td>
<td>810 ± 42</td>
<td>2780 ± 178</td>
</tr>
<tr>
<td>60 (12)</td>
<td>490 ± 27</td>
<td>240 ± 19</td>
<td>370 ± 49</td>
<td>4280 ± 656**</td>
<td>490 ± 41**</td>
<td>710 ± 35</td>
<td>2440 ± 144</td>
</tr>
<tr>
<td>120 (24)</td>
<td>430 ± 31*</td>
<td>230 ± 17</td>
<td>400 ± 22</td>
<td>2060 ± 522***</td>
<td>190 ± 36**</td>
<td>640 ± 49*</td>
<td>1890 ± 155*</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 6 rats/group. Values significantly different from the control value: * P < 0.05, ** P < 0.01, *** P < 0.001 (one-way analysis of variance).
The effect on the glycolytic intermediates

The concentrations of all the intermediates measured in spermatozoa from controls except fructose 1,6-bisphosphate increased during the incubation. Glycerate 3-phosphate and phosphoenol pyruvate did not accumulate in spermatozoa from rats treated with 6-chloro-6-deoxyglucose (120 µmol/kg/day) but the concentration of fructose 1,6-bisphosphate was higher in these cells than in controls and continued to increase during the incubation. The spermatozoa from rats treated with 6-chloro-6-deoxyglucose contained less glucose 6-phosphate than did the controls but the concentration of triose phosphate was not significantly different (Table 4).

Table 4. The concentration of some glycolytic intermediates in epididymal spermatozoa from rats given 6-chloro-6-deoxyglucose

<table>
<thead>
<tr>
<th>Dose µmol (mg)/kg/day</th>
<th>Incubation time (min)</th>
<th>Concentration (nmol/10⁸ spermatozoa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose 6-phosphate</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>55.7 ± 3.8</td>
</tr>
<tr>
<td>120 (24)</td>
<td>40</td>
<td>137.6 ± 17.3</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 3 observations except for glucose 6-phosphate (n = 6).
Values significantly different from the control value: *P < 0.05, **P < 0.01 (t test).
† Dihydroxyacetone phosphate + glyceraldehyde 3-phosphate.

Discussion

These data are not sufficient to prove conclusively that the inhibition of glucose oxidation in spermatozoa from rats treated with 6-chloro-6-deoxyglucose is the primary cause of their infertility. However, the observation that glucose oxidation by spermatozoa was inhibited by a lower dose of each 6-chloro-6-deoxysugar tested than was fertility supports this hypothesis. Moreover, the threshold dose for the two effects was altered in a parallel fashion when the structure of the hexose moiety was varied. 6-Chloro-6-deoxyfructose or 6-chloro-6-deoxyglucitol were the most potent contraceptives of the compounds tested and 6-chloro-6-deoxygalactose the least effective.

Rat spermatozoa produce no lactate when incubated under these conditions and the activity of the pentose phosphate pathway is probably very small since the activity of glucose 6-phosphate dehydrogenase is very low in these cells (Brooks, 1976; W. C. L. Ford & A. Harrison, unpublished observations). The oxidation of pyruvate by spermatozoa from rats treated with 6-chloro-6-deoxyglucose (120 µmol/kg/day for 8 weeks) was not impaired (Table 2). Therefore the production of ¹⁴CO₂ from [¹⁴C]glucose provides an index of flux through the glycolytic pathway and the metabolic lesion produced by the 6-chloro-6-deoxysugars is most probably located there. However, it should be borne in mind that more glucose was converted to glycolytic intermediates than to CO₂ under these conditions (Tables 1 and 2).

The assays of enzyme activities suggest that the site of action is triose phosphate isomerase or glyceraldehyde 3-phosphate dehydrogenase, as found for α-chlorohydrin and ram spermatozoa (Brown-Woodman, Mohri, Mohri, Suter & White, 1978). The concentrations of the glycolytic intermediates support this view because the concentrations of glyceralate 3-phosphate and phosphoenol-pyruvate did not increase in spermatozoa from treated rats during the incubation with glucose. However, the changes in glucose 6-phosphate and triose phosphate concentrations compared to the controls suggest that other factors are involved.
The enzyme activities reported here are broadly similar to those reported by Brooks (1976) with the exception of phosphofructokinase. The higher activity of this enzyme found in our study may be because we included the activator AMP in the assay medium. The observed enzyme activities are much in excess of the glycolytic flux and this is true even of glyceraldehyde 3-phosphate dehydrogenase in spermatozoa from rats treated with 6-chloro-6-deoxyglucose (120 μmol/kg/day).

The concentrations of glucose 6-phosphate, fructose 1,6-bisphosphate and triose phosphates in the spermatozoa are very high. It is possible that some of these compounds may leak out of the spermatozoa so that the intracellular concentrations are not as great as the data suggest. Human spermatozoa were permeable to glucose 6-phosphate (Peterson & Freund, 1969). Wherever they are located, the production of such a large quantity of phosphorylated intermediates must absorb a significant proportion of the ATP available to the cells and thus represent a large proportion of the flux through the early steps of the glycolytic pathway.

The 6-chloro-6-deoxysugars therefore appear to affect spermatozoa from treated rats in much the same way as does α-chlorohydrin and these results confirm that they have no effect on spermatogenesis. Unfortunately, although the acute toxicity of the 6-chloro-6-deoxysugars is much less than that of α-chlorohydrin, the chronic administration of high doses to mice produces side effects which preclude their use as contraceptives by man (Ford, 1980; Ford & Waites, 1980).

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References


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