

# Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat

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## Abstract

A study was undertaken to estimate the activities of the key enzymes of glycolysis, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle in purified rat spermatocytes and spermatids, which have been shown to die in glucose-containing medium and require lactate/pyruvate for maintaining normal ATP concentrations. The aim was to elucidate the changes in the glycolytic and oxidative potential of germ cells undergoing meiosis. Pachytene spermatocytes and round spermatids from adult rat testis were purified to ~90% purity by trypsin digestion followed by a combination of centrifugal elutriation and Percoll density gradient centrifugation. After the purity and viability of these cells had been established, their contents of hexokinase, phosphofructokinase, lactate dehydrogenase (LDH) and LDH-X of glycolysis, glucose 6-phosphate dehydrogenase of the pentose phosphate pathway and citrate synthase, aconitase, malate dehydrogenase and 2-oxoglutarate dehydrogenase of the TCA cycle were estimated. These enzymes were also estimated in epididymal spermatozoa for comparison with the testicular germ cells. The results indicate greater activity of glycolytic and pentose phosphate pathway enzymes in spermatocytes than in spermatids, which exhibited greater activity of TCA cycle enzymes than the former. The difference in activity was statistically significant for most of the enzymes studied. In contrast, spermatozoa exhibited markedly greater activity of glycolytic enzymes and significantly lower activity of pentose phosphate pathway and TCA cycle enzymes than did the testicular germ cells. We conclude that the unusual dependence of spermatids exclusively on lactate may be due to their lower glycolytic potential, whereas spermatocytes with comparatively greater glycolytic activity have an intermediate dependence on lactate and are therefore able to utilise lactate, pyruvate, or both, while retaining a better ability to utilise glucose. Spermatozoa with the greatest glycolytic potential and the lowest TCA cycle activity appear to be 'programmed' to utilise exclusively glucose/fructose for energy.

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## Introduction

Spermatogenesis is a highly synchronised, regular, continuous and extremely complex process of cellular differentiation by which a spermatogonial 'stem cell' is gradually transformed into a highly specialised haploid spermatozoon. The halving of the number of chromosomes in the process of spermatogenesis provides an interesting model for the investigation of a developmental process involving structural and metabolic changes in the germ cells. It is well known that the spermatogonia may utilise glucose as the major energy substrate (1), but spermatocytes and spermatids suffer a rapid decline in their ATP content in glucose-supplemented media and require lactate/pyruvate for the maintenance of their ATP concentrations (2, 3). In contrast, spermatozoa use glucose/fructose as the major source of energy (4). Therefore, it appears that, at each stage of spermatogenesis, there is a change in the substrate required for energy. However, to date no study

has thoroughly investigated the activities of the enzyme components of all the major metabolic pathways of energy metabolism of each germ cell type in the testis. The complexity of spermatogenesis has tended to discourage biochemical analysis of this process. The heterogeneous cell population of the testis makes the investigation of a single cell type difficult, and studies on whole testis preparations do not permit conclusions as to the enzyme content of specific populations of germ cells. Histochemical analysis has helped in the localisation of enzymes to specific cell types; however, for quantitative analysis it would be ideal to obtain homogeneous cell populations from testis (5). With the introduction of more modern techniques of cell purification, namely unit gravity sedimentation and centrifugal elutriation (6, 7), it is now possible to isolate and purify specific germ cell populations to near homogeneity for studies on their metabolism.

In the present study, we isolated and purified primary spermatocytes (meiotic germ cells) and round

spermatids (post-meiotic germ cells) from the testis of young adult rats to ~90% purity and estimated the activities of some key enzymes of glycolysis, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle in these cells, to investigate the changes that occur in the genetic expression of metabolic enzymes during the process of meiosis. In some earlier studies (1, 8, 9) the activities of glycolytic enzymes were estimated in enriched fractions of spermatocytes and spermatids; however, similar information is lacking on the enzymes of the TCA cycle, which apparently becomes more important during meiosis. Moreover, in the earlier studies, the activities were always expressed on the basis of the number of cells. Given that the spermatids are the result of two cell divisions and thus would have a smaller complement of cellular organelles (including mitochondria), expression of enzyme activities on the basis of cell numbers is not justified; specific enzyme activities per milligram of cell protein would give more meaningful data. We have therefore expressed all enzyme activities as specific activities. Epididymal spermatozoa (typical glucose-metabolising cells) have also been included in the study, as a reference for comparison with the testicular germ cell types. In addition, we have used centrifugal elutriation for purification of testicular germ cell types; not only is this a rapid method, but in addition the cells remain in isotonic medium during separation and are not exposed to the hypotonic and hypertonic solutions of a gradient.

## Materials and methods

### Animals

Young adult Sprague–Dawley rats weighing 180–200 g were obtained from the Institute colony. The animals were maintained in air-conditioned quarters ( $25 \pm 2^\circ\text{C}$ ) and fed with standard pellet diet. Water was available *ad libitum*.

### Chemicals

All the chemicals used were obtained from Sigma Chemical Company, St Louis, MO, USA. All solutions were prepared in triple glass distilled water.

### Purification of germ cells

Mid-pachytene spermatocytes and round spermatids were isolated by trypsin digestion and purified by centrifugal elutriation and density gradient centrifugation by the method of Meistrich *et al.* (7). Briefly, testes obtained from young adult animals were decapsulated, chopped and placed in Basal Medium Eagle (BME) containing trypsin (0.2%), glucose (0.1%) and DNase (17  $\mu\text{g}/\text{ml}$ ) at  $34^\circ\text{C}$  for 40 min with shaking in a shaker water bath. Trypsin action was stopped with soybean trypsin inhibitor (0.04%) in the presence of DNase

(2  $\mu\text{g}/\text{ml}$ ) and the sample was filtered through 100 mesh screen and glass wool and placed in FCS (8%) and DNase (2  $\mu\text{g}/\text{ml}$ ) in BME. The cells were later pelleted, suspended in BME (with DNase 2  $\mu\text{g}/\text{ml}$ ) and loaded into a Beckman Elutriator Rotor (JE-6B) with a standard chamber fitted on a Beckman High Speed Centrifuge (J2-21). Two fractions (I and II) were collected at 3000 r.p.m. at flow rates of 18.0 and 31.5 ml/min, and then the rotor speed was reduced to 2000 r.p.m. and another two fractions (III and IV) were collected at flow rates of 23.0 and 40.0 ml/min respectively. Fractions II and IV contained pachytene spermatocytes and round spermatids at purities of ~80% and 75% respectively. The two fractions were layered separately over linear gradients of 25–37% and 23–33% Percoll respectively and centrifuged at 11 000 *g* for 15 min in a Beckman swinging bucket (SW-41 Ti) rotor fitted on to a Beckman L8-M ultracentrifuge using slow acceleration and deceleration. The major band was recovered through a puncture in the side of the tube and diluted with BME. The cells were pelleted, suspended in BME and counted. After cell viability had been established by the trypan blue exclusion test, the cells were stored at  $-20^\circ\text{C}$  and the enzyme assays were completed the next day.

### Purity check

#### By cell morphology

A thin smear of purified cells was air-dried and fixed with Bouin's fluid. The cells were later stained with haematoxylin and examined under a microscope. Percent contamination by other cell types was determined.

#### By cell size

The purity of cell type was also examined by a Coulter Cell Counter (Model ZM) with Channelyzer II as described by Bucci *et al.* (6).

### Isolation of spermatozoa

Spermatozoa were isolated from cauda epididymis by giving it one or two cuts with fine scissors and gently teasing the tissue in BME. The spermatozoa were pelleted at 1000 *g* for 10 min, washed and suspended in fresh BME. Purity was checked by cell morphology and viability by dye exclusion, as detailed earlier.

### Enzyme assays

For all enzyme assays, the cells were thawed and homogenised at  $4.0^\circ\text{C}$  in an ice bath using Ultra Turrax (Janke & Kunkel, Staufen, Germany). The homogenising medium for glycolytic enzymes was 50 mmol/l triethanolamine buffer (pH 7.6) containing 1 mmol/l EDTA, 2 mmol/l  $\text{MgCl}_2$  and 30 mmol/l mercaptoethanol. The homogenate was centrifuged at  $4^\circ\text{C}$  for 30 min at

105 000 *g* and the supernatant was used for enzyme estimations. For TCA cycle enzymes, cells were homogenised in 0.1 mol/l potassium phosphate buffer (pH 8.0) containing 10 mmol/l EDTA, 0.1% sodium deoxycholate and 1% BSA. A supernatant obtained at 10 000 r.p.m. × 10 min was used for enzyme assays. The assay procedures followed have been detailed earlier (10, 11). All enzyme assays were performed in duplicate and the mean of the two was considered as one value; results are expressed as mean ± s.e. of five such values. A unit of enzyme activity was defined as 1 μmol substrate transformed/min at 30 °C. Results were analysed by Student's *t*-test and *P* values <0.05 were considered to be significant.

## Results

Trypsinisation of testicular tissue resulted in complete separation of testis cells (Fig. 1a); after elutriation, these were isolated into a spermatocyte (~80% mid-pachytene) fraction and a spermatid (~75% round spermatid) fraction. Density gradient centrifugation of spermatocyte and spermatid fractions gave >90% purity of the two cell types (Fig. 1b and c). The trypan blue exclusion test showed >95% viability of the purified cells in the two fractions. Isolated spermatozoa exhibited >95% purity and viability.

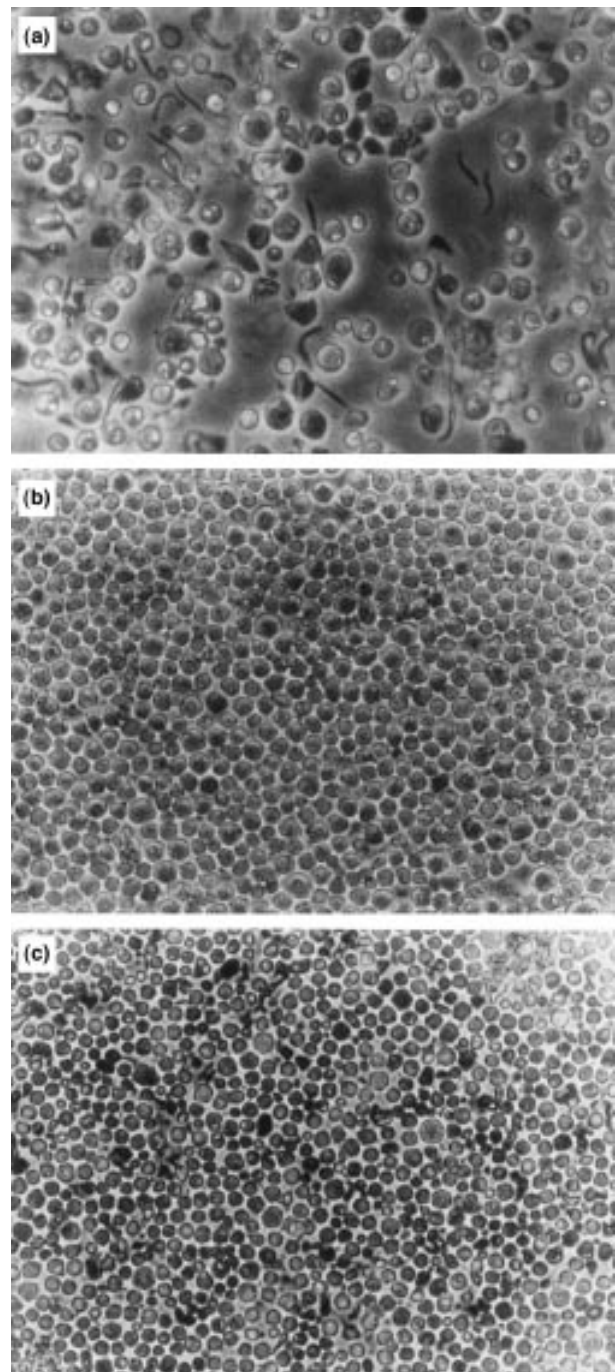
Figure 2 shows the activities of key enzymes of glycolysis (hexokinase, phosphofructokinase, lactate dehydrogenase (LDH) and LDH-X), the pentose phosphate pathway (glucose 6-phosphate dehydrogenase) and the TCA cycle (malate dehydrogenase, 2-oxoglutarate dehydrogenase, citrate synthase and aconitase), expressed as units/mg protein in purified spermatocytes, spermatids and spermatozoa of rat. Measurable activities of all these key enzymes were detected in spermatocytes, spermatids and spermatozoa, with significant differences between values in the different cell types.

### Glycolysis

The enzymes of the glycolytic pathway exhibited significantly greater activities in spermatocytes than in spermatids; the differences between their hexokinase, phosphofructokinase, LDH and LDH-X activities and those in spermatids were statistically significant ( $P < 0.05$ , 0.01, 0.05 and 0.05 respectively). Spermatozoa exhibited the greatest glycolytic activity; the difference between their hexokinase, phosphofructokinase, LDH and LDH-X activities and those of spermatids were statistically significant ( $P < 0.001$ , 0.001, 0.01 and 0.001 respectively). Phosphofructokinase showed the lowest activity, and LDH the highest (Fig. 2).

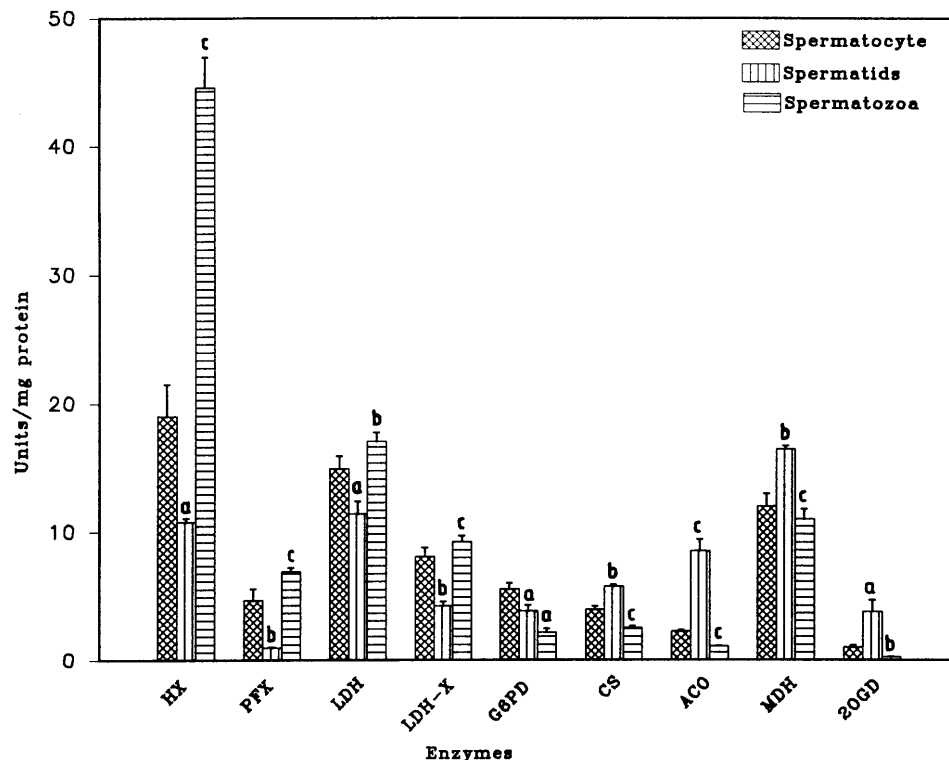
### Pentose phosphate pathway

As in the case of the enzymes of glycolysis, the enzymes of the pentose phosphate pathway also showed



**Figure 1** (a) Unseparated cells; (b) ~90% pure pachytene spermatocytes; (c) ~90% pure round spermatids. Original magnification: ×600.

significantly greater activity in spermatocytes than in spermatids. The enzyme glucose 6-phosphate dehydrogenase showed ~20% greater activity in spermatocytes and the difference from that in spermatids was statistically significant ( $P < 0.05$ ); the activity was



**Figure 2** Specific activities (mean  $\pm$  s.e. of five values) of carbohydrate metabolising enzymes in purified spermatocytes, spermatids and spermatozoa. HX, hexokinase; PFX, phosphofructokinase; LDH, lactate dehydrogenase; LDH-X, lactate dehydrogenase-X; G6PD, glucose 6-phosphate dehydrogenase; CS, citrate synthase; ACO, aconitase; MDH, malate dehydrogenase; 2OGD, 2-oxoglutarate dehydrogenase. <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$  ( $P$  values are for comparisons of spermatids with spermatocytes, and spermatozoa with spermatids).

further significantly reduced in spermatozoa ( $P < 0.05$ ) compared with spermatids (Fig. 2).

### TCA cycle

The enzymes of TCA cycle exhibited lower activities in spermatocytes than in spermatids. Malate dehydrogenase, 2-oxoglutarate dehydrogenase, citrate synthase and aconitase showed greater activities in spermatids and the differences from the activities in spermatocytes were statistically significant ( $P < 0.01$ , 0.05, 0.01 and 0.01 respectively). Spermatozoa exhibited the lowest TCA cycle activity and, compared with activities in spermatids, significantly lower activities of citrate synthase ( $P < 0.001$ ), aconitase ( $P < 0.001$ ), malate dehydrogenase ( $P < 0.001$ ) and 2-oxoglutarate dehydrogenase ( $P < 0.01$ ) were detectable in spermatozoa. Malate dehydrogenase showed the greatest activity, and 2-oxoglutarate dehydrogenase showed the lowest (Fig. 2).

### Discussion

The activities of key enzymes of the major metabolic pathways of carbohydrate metabolism give a clear indication that germ cells undergoing meiosis exhibit

a change in the type of substrate required for their energy metabolism. The equilibrium between glycolysis and the TCA cycle shifts more in favour of oxidative metabolism in post-meiotic germ cells. Thus the ratio of glycolysis to TCA cycle activity is likely to be greater in spermatocytes than in spermatids. However, in spermatozoa the equilibrium again shifts drastically in favour of glycolysis.

Nakamura *et al.* (9) previously measured the activities of glycolytic enzymes in enriched fractions of rat spermatocytes and spermatids, and showed markedly lower activities in spermatids than in spermatocytes. We also found significantly lower activities of glycolytic enzymes in spermatids than in spermatocytes. However, the vast difference in activities between the two cell types reported earlier (8, 9) was due to the basis on which the investigators had expressed the enzyme activities – namely that of cell numbers. As explained in the introduction, a more accurate measure of the reduction in glycolytic potential during meiosis can be obtained by expressing activities on the basis of milligrams of cell protein, rather than cell number. However, our values can be converted approximately into units/ $10^6$  cells by dividing by a factor of 9.7 for spermatocytes, 12.5 for spermatids and 8.8 for spermatozoa.

The lack of earlier information on the activities of TCA cycle enzymes in spermatocytes and spermatids is surprising, as the involvement of this pathway in the production of ATP by the two cell types has always been considered to be very important (3, 12, 13). In the present study, our finding of a significant increase in TCA cycle enzyme activities in post-meiotic germ cells (spermatids) compared with those in spermatocytes clearly indicates a preference for the TCA cycle over glycolysis in spermatids. This observation is supported by the earlier finding that respiratory inhibitors reduce pyruvate/lactate consumption by spermatocytes, whereas spermatids degenerate completely in their presence (13).

Both spermatocytes and spermatids have been shown to suffer a rapid decline in their ATP concentrations in glucose-supplemented media (14–16) and, whereas lactate alone was able to restore the ATP concentrations of spermatids (16), lactate and pyruvate were equipotent in maintaining normal ATP concentrations of spermatocytes (1). Isolated rat spermatocytes, however, survived for at least 24 h in the presence of glucose (17). If this is the case, then the spermatocytes are half-way towards becoming dependent on lactate. Thus spermatocytes retain a better ability to oxidise glucose, as is indicated by their higher activities of glycolytic enzymes compared with spermatids.

The germ cells have some pentose phosphate pathway activity, as indicated by glucose 6-phosphate dehydrogenase activity. As it is a cytosolic enzyme, glucose 6-phosphate was naturally found to have a greater activity in spermatocytes. This pathway is required to meet the needs of biosynthesis of nucleotides for RNA by generating important substrates such as NADPH and ribose 5-phosphate. Very little is known about the synthesis of nucleotides and purine salvage reactions in spermatogenic cells (18).

Germ cells in mammalian testis develop in a tubular environment that is controlled to a high degree by Sertoli cells. The fluid of the seminiferous tubules has been found to be rich in lactate and very poor in glucose and pyruvate (19, 20). Thus spermatocytes with sufficient potential to utilise glucose and pyruvate may actually be utilising mostly lactate as the substrate available in bulk *in situ*. Isolated germ cells, however, consume oxygen at the maximum rate in the presence of both lactate and glucose (2). In contrast, critical dependence of round spermatids on the TCA cycle and electron transport system for energy is indicated by the inhibition of their ATP synthesis with lactate as substrate in response to the addition of inhibitors of alpha-keto acid dehydrogenase or retinol, rutamycin, 2,4-dinitrophenol etc. (3). Lactate also stimulated RNA and protein synthesis in spermatids (2). Early studies by Free (21) had shown that carbohydrates are an exceedingly important energy source for mammalian testis. Sertoli cells incubated in glucose-containing medium under aerobic conditions convert glucose to

lactate (22, 23), which is secreted into the medium. The lactate secreted by the Sertoli cells is stimulated by follicle-stimulating hormone (24, 25) and is directed towards the adluminal compartment. It is very likely that the rate of glycolysis and lactate production *in situ* is under fine regulation to maintain a steady-state concentration of lactate in the spermatogenic micro-environment (26). Thus the survival of the advanced germ cells in testis is strictly dependent on carbohydrate metabolism, including both anaerobic (glycolysis) and aerobic (TCA cycle) pathways. The sugars are actively glycolysed by the Sertoli cells, which secrete lactate as the major energy substrate for spermatocytes and spermatids.

The germ cells use the TCA cycle preferentially over glycolysis; however, all the testicular germ cells do not rely on oxidative metabolism: spermatogonia may depend more on glycolysis, spermatocytes are intermediate and can depend to some extent on glycolysis, whereas spermatids use the TCA cycle exclusively for their energy. However, interestingly, spermatozoa again regain the ability to utilize glucose/fructose (27). Therefore, during spermatogenesis, the dependence of germ cells on lactate/pyruvate and glucose for energy metabolism keeps changing, but the advantage of this process remains an enigma.

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