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Activity of *myo*-inositol-1-phosphate synthase in the epididymal spermatozoa of rams

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Summary. Studies of six 8–9-month-old rams showed that the specific activities of *myo*-inositol-1-phosphate synthase (EC 5.5.1.4) were highest in epididymal spermatozoa, intermediate in testis and lowest in epididymal tissue. The activity per spermatozoon decreased from caput to cauda. The levels of activity of *myo*-inositol-1-phosphate synthase in ejaculated spermatozoa from four 3-year-old rams and the seminal vesicles of two 3-year-old rams were insignificant, but in pooled Sertoli cells from the testes of young lambs, the specific activity was much lower than in epididymal spermatozoa although activity per cell was of the same order of magnitude. We conclude that epididymal spermatozoa contain a significant, if not the major, amount of *myo*-inositol-1-phosphate synthase activity of the epididymis.

Introduction

Free *myo*-inositol is one of the major constituents in fluids of the mammalian reproductive tract (Hartree, 1957; Mann, 1964; Eisenberg & Bolden, 1964; Setchell, Dawson & White, 1968). Dietary deficiency of this factor in hamsters has been associated with reproductive disturbances (Hamilton & Hogan, 1944) and it has been suggested by several workers that it may play a part as an osmoticum (Mann, 1964). More recently phosphatidyl inositol has been shown to be higher in testicular than in ejaculated spermatozoa and to be involved in the maturation process of the spermatozoa (Scott, Voglmayr & Setchell, 1967). Scott & Setchell (1968) reported uptake of D-[U-¹⁴C]glucose into the monophosphatidyl inositol of testes but not into testicular spermatozoa of rams. Voglmayr & White (1971) reported that 65–75% of the label which accumulated in ram testicular spermatozoa incubated with D-[U-¹⁴C]glucose was *myo*-inositol, but in ejaculated spermatozoa only trace amounts were detectable.

The enzymes responsible for synthesis of *myo*-inositol, namely *myo*-inositol-1-phosphate synthase (EC 5.5.1.4), referred to hereafter as synthase, and 1L-*myo*-inositol-1-phosphatase (EC 3.1.3.25) were first isolated from rat testis by Eisenberg & Bolden (1963). The first of these enzymes converts D-glucose 6-phosphate to 1L-*myo*-inositol 1-phosphate (IUPAC/IUB, 1973) and the latter to free *myo*-inositol (Eisenberg, 1967). Robinson & Fritz (1979) found the highest specific activity for synthase in whole epididymal tissue of rats and about one-tenth as much in testes and Sertoli cell-enriched preparations. They found no detectable activity in spermatozoa of the epididymis or in testicular spermatocytes and spermatids. Roberts, Scouten & Nyquist (1976) reported that incorporation of D-[¹⁴C]glucose into *myo*-inositol was greatest in immature spermatozoa–cytoplasmic droplet complexes and immature droplets; incorporation decreased with maturation of these cells.

We have observed unexpectedly high synthase activity in samples of epididymal spermatozoa and to assess the importance of this activity we have measured and compared the level of activity of *myo*-inositol-1-phosphate synthase in various tissues of rams.

Materials and Methods

Chemicals

D-[1-¹⁴C]Glucose 6-phosphate was obtained from New England Nuclear Corp., Boston, Massachusetts. Ham's F12 medium was from Grand Island Biological Co., Grand Island, New York, calf serum from Sterile Systems, Inc., Logan, Utah, testosterone from Sigma Chemical Co., St Louis, Missouri, and FSH from the National Pituitary Agency, NIAMDD, NIH, Bethesda, Maryland.

Tissues

The testes and epididymides of six 8- to 9-month-old rams (Western range sheep, a cross between Panama and Suffolk) were dissected out. The testes were frozen immediately. The epididymides were separated arbitrarily into caput, corpus and cauda (41 ± 2 , 15 ± 4 and $44 \pm 2\%$, respectively, of the total weight of the epididymis) and immersed in a medium containing 0.02 M-Tris-acetate (pH 7.4), 0.154 M-KCl and 0.2 mM-dithiothreitol. Numerous incisions were made in each section to cut the tubule and allow the release of spermatozoa. After 30 min, the tissue was lifted from the sperm suspension and frozen. Spermatozoa in the washing buffer were estimated by counting an aliquant in a haemocytometer. From the volume, the total number of spermatozoa washed from each section was calculated. The total numbers of spermatozoa recovered from the caput, corpus and cauda of the epididymis were in the range of 10^8 , 10^8 and 10^9 cells per section, respectively. These figures represent only a fraction of the spermatozoa in the epididymis, the rest remaining with the tissue. Microscopic inspection showed only spermatozoa in the suspensions. The cytoplasmic droplets were proximal in caput spermatozoa, translocating in corpus spermatozoa and distal or missing (approximately 50%) in cauda spermatozoa. Spermatozoa were separated from the washing buffer by centrifugation and both fractions were frozen. Enzyme isolations were completed within 3 days after slaughter and the same procedure was followed for seminal vesicle and ejaculated spermatozoa from adult rams and testis from young lambs.

Sertoli cells

Testes from two 2.5- to 3-week-old ram lambs were excised and placed in Hanks' basic salt solution (pH 7.4) at 4°C. The tissue was prepared for cell culture essentially by the methods of Dorrington & Fritz (1975) with the modifications of Wilson & Griswold (1979), except that the incubation time in trypsin was 40 min and in collagenase 30 min. During enzyme digestion, the tissue was vigorously agitated by hand every 5–10 min. Washing the tissue through a grid has been discontinued. After completion of the collagenase digestion and the washes, the tissue was allowed to settle in a 50-ml polycarbonate tube before the final pellet was obtained. Tubule fragments were placed in culture in Ham's F-12 medium, supplemented with 1% normal calf serum, testosterone (2 µg/ml) and ovine follicle-stimulating hormone (4 ng NIH-FSH-S13/ml). The cultures were incubated at 37°C in a 5% CO₂ in air atmosphere for 6 days. The medium was changed every 2 days. Cells from both animals were pooled for enzyme isolation to obtain measurable amounts of enzyme.

Isolation of enzyme

Epididymal and testis tissues were thawed and homogenized in 10 volumes of Tris-acetate buffer. For the testes, a cross-section was cut for homogenization. More than 80% of spermatozoa were disrupted by freezing and thawing twice. Homogenates and broken spermatozoa were

centrifuged at 27 000 *g*. Sperm counts were taken after homogenization to determine the percentage of unbroken cells remaining and this figure was used in calculating total activity and mg protein. Protein which precipitated from homogenates in 30–60% saturated ammonium sulphate was taken up in 0.02 M-Tris-acetate (pH 7.4), dialysed for 3 h with three changes of buffer and tested for activity. Preliminary experiments showed that >90% of the activity occurred in this fraction. The heat step recommended by Eisenberg (1967) to remove phosphatase was found to destroy 80% of the synthase in the broken sperm suspension when compared to that recovered from ammonium sulphate precipitates of unheated suspensions. This step was omitted. Analysis of reaction mixtures after incubation showed <12% hydrolysis of glucose 6-phosphate by endogenous phosphatases, but this decrease did not affect the activity since the enzyme was assayed at saturating substrate concentration. All preparations were assayed using the same time schedule so as to be comparable. The protein content of the fractions was determined by the Coomassie Blue method (Bradford, 1976).

Assay

Preliminary tests showed that the ram testicular preparation had an optimum pH of 7.4 and the assay was carried out at 30°C in a 0.5-ml volume containing 2.4 mM-glucose 6-phosphate (containing 0.5 μ Ci [$1\text{-}^{14}\text{C}$]glucose 6-phosphate, 1 mM-NAD⁺, 5 mM-(NH₄)₂SO₄, and 40 mM-Tris-acetate (pH 7.4). At 2.5 h, when the reaction was still linear, the incubations were terminated by boiling. Inositol was isolated and determined as described elsewhere (Loewus, 1977). The activities reported represent averages of duplicate determinations. The deviation from the average was <12%.

Results

Distribution of synthase activity

Of the total synthase activity isolated from the testis, epididymal tissue (plus any remaining spermatozoa), and epididymal spermatozoa, 96% occurred in the testis due to its large weight (average, 190 \pm 38 g). The synthase activity in the testes contained 0.225 \pm 0.122 nmol \cdot min⁻¹ \cdot g⁻¹ wet weight of tissue. The next largest fraction, 2.5% (0.036 \pm 0.018 nmol \cdot min⁻¹ \cdot g⁻¹ wet weight of tissue), occurred in epididymal tissue plus unflushed spermatozoa, and 1.3% was found in the epididymal spermatozoa. The total activity in spermatozoa that were washed from the epididymides varied from 22 to 42% of the combined activity of epididymal tissue and spermatozoa. About 0.2% was found in the buffer that was used to wash spermatozoa from the epididymal sections. It had a specific activity only 0.4% of that of the spermatozoa. Synthase activity from ejaculated spermatozoa of four 3-year-old rams was barely detectable, 1–2% of the activity of epididymal spermatozoa.

Specific activity of fractions

The specific activities of the enzyme (Table 1) were highest for epididymal spermatozoa, 6- to 9-fold that found for the testis and more than 10-fold the specific activity of the enzyme isolated from the epididymal tissue and unflushed spermatozoa. The activity per mg protein for spermatozoa did not differ significantly between caput, corpus, and cauda cells. The activity per cell for corpus and cauda was about one-third of that of the caput value.

Sertoli cells

Attempts to culture sufficient Sertoli cells from the testes of 6 animals were unsuccessful. Sertoli cells from adult animals frequently exhibit very poor attachment to tissue culture dishes and are difficult to maintain in a metabolically stable state. Sertoli cells were successfully cultured from the

Table 1. Specific activity of *myo*-inositol-1-phosphate synthase in whole tissue and spermatozoa of the ram reproductive tract

Source	Synthase activity	
	nmol·min ⁻¹ ·mg ⁻¹ (protein)	nmol·10 ⁻⁹ ·min ⁻¹ cell ⁻¹
Epididymal spermatozoa		
Caput	0.086 ± 0.035 ^a	0.466 ± 0.337 ^a
Corpus	0.083 ± 0.027 ^a	0.148 ± 0.051 ^b
Cauda	0.149 ± 0.116 ^a	0.151 ± 0.161 ^b
Tissue (whole)		
Testis	0.015 ± 0.007 ^b	—
Epididymal tissue (plus unflushed spermatozoa)		
Caput	0.004 ± 0.002 ^c	—
Corpus	0.006 ± 0.005 ^c	—
Cauda	0.010 ± 0.004 ^b	—

Values are mean ± s.d. for observations on 6 rams. Within columns, values with different superscript letters were significantly different, $P < 0.005$ (Fisher's least significant difference test for the transformed variable, \log_{10} activity).

testes of two 2- to 3-week-old lambs, pooled to obtain sufficient material. The specific activity, while measurable, was only 0.008×10^{-3} nmol·min⁻¹·mg⁻¹ (protein), i.e. 0.01% of that of spermatozoa. The testes from which these were cultured yielded synthase with a specific activity of 0.005 nmol·min⁻¹·mg⁻¹ (protein), one-third that reported for the adult animals. However, the activity per Sertoli cell was 0.821×10^{-9} nmol·min⁻¹, comparable to that of spermatozoa. The significance of this would require more data to evaluate.

Seminal vesicle

The synthase activity isolated from the seminal vesicles of two 3-year-old rams was too low to be significant.

Discussion

The high specific activity of the enzyme isolated from epididymal spermatozoa, together with the fact that 22–42% of the activity segregated with the spermatozoa washed from the epididymis, suggests that these cells are the site of a major part of *myo*-inositol-1-phosphate synthesis in the epididymis (and, presumably, of its hydrolysis to free *myo*-inositol). The activity remaining in the epididymal tissue may be due to remaining spermatozoa but an independent synthesis by the epididymal tissue is not precluded by these experiments. Contrary to these findings, Eisenberg & Bolden (1964) and Robinson & Fritz (1979) found no synthase activity in epididymal spermatozoa of rats. However, the activities we have found in ram tissues are below the level of detectability of the assay used by Robinson & Fritz (1979) and these authors also included a heating step which we found to destroy sperm synthase.

Roberts *et al.* (1976) reported that inositol synthesis decreases with the in-vitro maturation of epididymal spermatozoa. When the total synthase per cell is considered, the present results show that it decreased from caput to cauda and was absent in ejaculated spermatozoa. Since this significant decrease was evident in the spermatozoa from the corpus epididymidis, which had not lost their cytoplasmic droplets, yet was not lower in cauda spermatozoa, half of which had lost droplets, the synthase cannot be presumed to be in the droplet. Synthase activity per mg protein

does not decrease because the protein isolated from cauda and corpus cells decreased also. The specific activities of the different spermatozoa, reported with respect to protein, do not differ outside the variation for different animals.

Pooled Sertoli cells from 2 young lambs had a negligible activity per mg protein, compared to spermatozoa, although the activity per cell was comparable. If the level of activity per mg protein in ram Sertoli cells had been anywhere near that reported for rat tissue by Robinson & Fritz (1979) (10000 times higher than reported here for ram Sertoli cells), it would have been observed even in the small amounts of Sertoli cells obtained from mature animals.

Our failure to find synthase activity in the vas deferens of lambs agrees with the results of Lewin, Yannai & Kraicer (1978) who found that the rat vas deferens concentrated *myo*-inositol from the blood.

The bulk of *myo*-inositol synthesis occurs in the testis. Voglmayr & Amann (1973) have shown in bulls and rabbits an uptake by the epididymis of 99% of the inositol coming from the testis and that a large part of this may be due to phosphatidylinositol synthesis in the maturing spermatozoa. We have now demonstrated the presence of *myo*-inositol-1-phosphate synthase in epididymal spermatozoa of rams, although it has been reported to be absent from rat epididymal spermatozoa (Eisenberg & Bolden, 1964; Robinson & Fritz, 1979). This activity may be necessary for supplementation of inositol reserves from the testis. In view of the findings of Voglmayr & White (1971) on *myo*-inositol synthesis by testicular spermatozoa, the possibility should be examined that the spermatozoa and spermatids may be the source of inositol synthesis in the testis and that the enzyme remains in the spermatozoa of the epididymis but disappears as they mature.

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