

SYNTHESIS AND METABOLISM OF MYOINOSITOL IN TESTICULAR AND EJACULATED SPERMATOZOA OF THE RAM

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Summary. The synthesis of inositol from glucose by ram testicular and ejaculated spermatozoa has been examined. During incubation for 2 hr under air, the intracellular accumulation of substrate carbon accounted for 10.2% and 2.3% of the glucose utilized by testicular and ejaculated spermatozoa respectively. Some 65 to 75% of the substrate carbon accumulating in testicular spermatozoa was inositol, whereas in ejaculated spermatozoa only trace amounts of the cyclitol were detectable. When incubated without exogenous substrate, testicular spermatozoa utilized intracellular inositol and accumulated labelled metabolites. The addition of extracellular inositol failed to stimulate the respiration of testicular or ejaculated spermatozoa above endogenous levels although small amounts of the cyclitol were oxidized. Rete testis fluid increased the oxygen uptake of testicular and ejaculated spermatozoa and increased the uptake of substrate carbon from inositol present in the fluid. It is suggested that the cytoplasmic droplet may be the site of inositol synthesis in the testicular spermatozoa.

INTRODUCTION

An active system in the mammalian testis for synthesis of myoinositol has been reported by Eisenberg & Bolden (1963) who also showed that the testis itself stores little inositol, while the epididymis, which possesses only a slight capacity for synthesis, maintains a much higher concentration of this cyclitol (Eisenberg & Bolden, 1964). It has, therefore, been suggested that inositol may play some rôle in the maturation of spermatozoa during their passage through the epididymis. Inositol occurs in ram rete testis fluid at a concentration over 100 times that in peripheral blood plasma (Wales, White, Scott & Wallace, 1961; Setchell, Dawson & White, 1968) and most of it is probably formed in the seminiferous tubules from glucose through the intermediate compounds, glucose 6-phosphate and D-myoinositol-1-phosphate (Eisenberg, 1967).

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Although Eisenberg & Bolden (1964) considered the possibility that spermatozoa are the site of synthesis, they were unable to demonstrate the formation of inositol by epididymal spermatozoa. However, epididymal or ejaculated spermatozoa differ from testicular spermatozoa in that the latter utilize more glucose than can be accounted for as carbon dioxide, lactic acid and amino acids (Voglmayr, Scott, Setchell & Waites, 1967; Murdoch & White, 1968) or incorporation into lipid (Scott, Voglmayr & Setchell, 1967). These results, together with the foregoing considerations, prompted us to examine the possible synthesis of inositol from glucose and its metabolism by testicular spermatozoa.

MATERIALS AND METHODS

Preparation and incubation of sperm suspensions

Testicular spermatozoa were collected from six mature Merino rams by the technique described elsewhere (Voglmayr *et al.*, 1967; Voglmayr, Larsen & White, 1970). Ejaculated spermatozoa were obtained after electrical stimulation (Blackshaw, 1954). The washed spermatozoa (Voglmayr *et al.*, 1967) were resuspended in calcium-free Krebs-Ringer, buffered with 20 mM phosphate (final concentration) to pH 7.3 (Umbreit, Burris & Stauffer, 1959), and incubated at 37° C for 2 hr in 5- or 15-ml Warburg flasks which were shaken at 60 cycles/min. The final volume of the reaction mixture was 1 ml (small flasks) and 3 ml (large flasks) and contained 1.5 to 2.0 and 12 to 20 × 10⁴ spermatozoa respectively.

[U-¹⁴C]D-glucose and [U-¹⁴C]myoinositol were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, and purified by chromatographic procedures (impurities <0.03%). The labelled substrates were added to the flasks with appropriate carrier compounds to give a final concentration of 3600 μg and 8 μCi/3 ml or 900 μg and 1 μCi/ml.

Alternatively, washed spermatozoa were incubated in cell-free rete testis fluid to which 1 μCi inositol was added to yield a final concentration of about 900 μg/ml.

Extraction and fractionation of intracellular compounds

At the completion of the incubation, the spermatozoa were separated from the suspending medium by density gradient centrifugation (Wales & O'Shea, 1966; Wales & Humphries, 1969) using 6 ml of the isotonic lactose rinse solution to which unlabelled glucose or inositol were added at the concentration of the incubation media. Aliquots of the sperm-free medium at the top of the isotonic lactose were carefully transferred into centrifuge tubes and deproteinized with 0.5 vol of 5% (w/v) ZnSO₄·7H₂O and 0.5 vol of 0.3 N-Ba(OH)₂. The neutral filtrates were stored for chemical analyses together with those obtained from aliquots of identically treated sperm suspensions before incubation.

The spermatozoa were resuspended in 1 ml of water and extracted with an equal volume of 5% (w/v) perchloric acid at 1° C. After centrifugation, the supernatant was transferred into centrifuge tubes and the precipitate extracted again. The pooled supernatants were neutralized with potassium hydroxide and, after storage at -10° C, the potassium perchlorate was removed by centri-

fugation at 1° C. Alkaline salts and multivalent cations were eliminated by passage through a column of Amberlite IRC-50 bed ion exchange resin (Rohm & Haas Company, Philadelphia, Pennsylvania, U.S.A.). The volume of the effluent was reduced by evaporation, the pH adjusted to 7.0 and aliquots applied to Whatman No. 1 filter paper which was saturated with volatile buffer (pH 3.6) consisting of pyridine:acetic acid:water, 1:10:89 by vol (Dawson, 1960). The perchloric acid-soluble intracellular compounds were resolved by high voltage ionophoresis (2000 V) during 55 min under water-cooled toluene, using an apparatus similar to that described by Ryle, Sanger, Smith & Kitai (1955).

Identification of myoinositol

After localization by autoradiography, each individual compound was extracted twice over 16 hr, each time with 10 ml of water. The volume of the eluates was reduced to 1 ml under vacuum and radioactivity in the eluates was assayed by liquid scintillation techniques. One compound, which appeared near the origin on the ionophoretogram, together with residual glucose, possessed over 75% of the total radioactivity. It was resolved by paper chromatography in (A) n-propanol:water:ammonia (7:2:1 by volume), (B) isopropanol:pyridine:acetic acid:water (8:8:1:4), (C) n-propanol:ethyl acetate:water (7:1:2), (D) n-propanol:ammonia:water (6:3:1) and (E) n-butanol:acetic acid:water (5:1:2). Since this isolated compound appeared on the chromatograms as a radioactive peak conterminous with a $\text{AgNO}_3/\text{NaOH}$ reactive spot (Trevelyan, Proctor & Harrison, 1950) with a mobility similar to authentic myoinositol, it was eluted and recrystallized with carrier inositol using 8.5 vol of methanol. Quantitative estimation of the radioactive inositol was made by the method described by Gaitonde & Griffiths (1966) after isolation in solvent system B and eluting the area corresponding to inositol.

The compound was further identified by gas liquid chromatography. Acetate derivatives of the cyclitol were prepared by the method of Sawardeker, Sloneker & Jeanes (1965) and resolved on a 6-ft column ($\frac{1}{8}$ in. inner diameter) of ECNSS-M (10% w/w) on 60- to 100-mesh Embacel with a gas chromatograph (Aerograph 200 series, Wilkins Instrument and Research Inc., Walnut Creek, Calif., U.S.A.). The chromatograph was equipped with a stream splitter, which allowed about half of the effluent gas containing ^{14}C -labelled myoinositol to pass directly into the flame detector, while the other half was diverted away and the radioactive ester collected into tubes containing anthracene for radioactive assay (Karmen, Giuffrida & Bowman, 1962). The standard used was inositol acetate prepared from purified inositol of known specific activity.

Metabolism of intracellular inositol

The testicular spermatozoa were separated from the incubation medium after incubation with [^{14}C]glucose for 2 hr. The spermatozoa were washed twice to remove residual glucose (about 5% of the radioactivity in the inositol fraction) and lactose and resuspended in Krebs-Ringer phosphate with no added substrate. The sperm suspension was divided in half; one portion was

extracted immediately, while the other half was incubated under air for 5 hr together with washed control spermatozoa which had not been pre-incubated with glucose. The metabolites accumulating intracellularly were separated and their radioactivity determined by liquid scintillation techniques.

Other analytical procedures and assay of radioactivity

Oxygen uptake was measured by manometry. The amount of substrate oxidized was calculated from assay of $^{14}\text{CO}_2$ trapped in 20% (w/v) KOH in the centre well of the Warburg flask. Glucose and lactic acid were determined by enzymatic methods (Huggett & Nixon, 1957; Barker & Britton, 1957) and by the chromatographic procedures (solvent system B) used for amino acid assay (Freinkel, Cohen, Arky & Foster, 1965).

TABLE 1

THE ACCUMULATION OF SUBSTRATE CARBON FROM $[\text{U-}^{14}\text{C}]$ GLUCOSE BY TESTICULAR AND EJACULATED RAM SPERMATOOZA

Spermatozoa	Oxygen uptake (μl)	Glucose utilized (μatoms substrate C)	Extracellular			Intracellular	
			Glucose oxidized (μatoms substrate C)	Lactic acid accumulated (μatoms substrate C)	Glucose converted to amino acids (μatoms substrate C)	Inositol (μatoms substrate C)	Unidentified compounds (μatoms substrate C)
Testicular	260 ± 26.10	24.00 ± 3.18	5.95 ± 0.28	10.92 ± 3.13	1.33 ± 0.46	1.76 ± 0.27	0.69 ± 0.11
Ejaculated	416 $\pm 40.89^{**}$	81.45 $\pm 5.85^{**}$	15.85 $\pm 1.53^{**}$	50.80 $\pm 2.86^{**}$	†	†	1.92 $\pm 0.19^{**}$

Values are the means \pm S.E. of six (testicular) and four (ejaculated) replicates and are calculated/10⁹ spermatozoa. The glucose utilized equals initial minus final amount; the glucose oxidized has been calculated from $^{14}\text{CO}_2$ trapped in 20% (w/v) KOH in centre well; lactic acid was calculated from [^{14}C]lactate present in the medium at the end of incubation; amino acids were assayed from radioactive peaks on the chromatogram with a mobility similar to aspartic acid, glutamic acid ($R_F = 0.08$ to 0.12) and other amino acids between the origin and glucose ($R_F = 0.58$).

** Highly significantly different from testicular spermatozoa ($P < 0.01$).

† Trace amounts.

Assay of radioactivity was made using a scintillator consisting of toluene-Triton X100 detergent (2:1) which contained 0.4 (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl) benzene (Patterson & Greene, 1965). Radioactivity was measured in an automatic liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill., U.S.A.) and all counts were corrected for background and efficiency of counting.

Statistical analysis

The statistical significance of the results has been assessed by analyses of variance (Tables 1 and 2). For some results, only the means \pm S.E. are given and the significance between means has been determined by paired *t*-tests.

TABLE 2

METABOLISM OF EXTRACELLULAR [U-¹⁴C]INOSITOL BY TESTICULAR (T) AND EJACULATED (E) RAM SPERMATOZOA IN RETE TESTIS FLUID (RTF) AND KREBS-RINGER PHOSPHATE (KRP)

Diluent	Oxygen uptake (μl/10 ⁹ cells)		Carbon dioxide from substrate carbon (μatoms/10 ¹⁰ cells)		Intracellular accumulation of substrate carbon (μatoms/10 ¹⁰ cells)	
	T	E	T	E	T	E
RTF†	262	402	13.8	3.0	14.9	9.1
KRP†+inositol (900 μg/ml)	181	166	4.3	6.6	8.6	††
KRP	177	185	—	—	—	—

Values are the means of four replicates; †† trace only in some replicates. The carbon dioxide formed has been calculated from ¹⁴CO₂ trapped in 20% (w/v) KOH in centre well. The radioactivity trapped in the centre well or accumulating in the sperm plug from reaction mixtures containing boiled (10 min) spermatozoa has been deducted in each case.

† Both diluents contained 1 μCi inositol/ml.

Summary of the analyses of variance

Source of variation	Variance ratios		
	Oxygen uptake	Carbon dioxide from substrate carbon	Intracellular accumulation of substrate carbon
A. Spermatozoa	16.40** (1)	6.87* (1)	17.11** (1)
B. Diluent	81.10** (2)	2.89 (1)	19.09** (1)
C. Replicate	2.14 (3)	2.35 (3)	1.49 (3)
Interaction A × B	16.74** (2)	14.79** (1)	0.66 (1)
Residual (error)	804 (15)	11.79 (9)	12.10 (9)

* *P* < 0.05; ** *P* < 0.01.

The degrees of freedom are shown in parentheses.

RESULTS

Dissimilation of glucose by testicular and ejaculated spermatozoa

The pattern of glucose dissimilation by testicular spermatozoa was in marked contrast to that of ejaculated cells (Table 1). The respiratory activity of testicular spermatozoa was less than in ejaculated spermatozoa and oxidation of added glucose accounted for a smaller percentage of the oxygen uptake in the former than in the latter cell type.

Testicular spermatozoa utilized less glucose and accumulated less lactic acid in the medium than did ejaculated cells but converted about 5% of the utilized glucose to amino acids. In contrast to these findings, only trace amounts of amino acids were detectable in the incubation medium of ejaculated spermatozoa.

While the total substrate carbon accumulating intracellularly in testicular spermatozoa was only about 28% greater than in ejaculated cells, in testicular spermatozoa it accounted for a much greater percentage of the substrate utilized (10% versus 2.3%). Further, there were differences between the two types of spermatozoa in the nature of the intracellular compounds containing substrate carbon. Some 65 to 75% of the intracellular substrate carbon in testicular spermatozoa was inositol; in addition, small amounts of the cyclitol accumulated extracellularly. By contrast, in ejaculated spermatozoa almost all the intracellular substrate carbon accumulated as metabolites other than inositol.

That the radioactive compound isolated from testicular spermatozoa is, in fact, inositol relies on the following findings. The compound always appeared with a mobility similar to authentic myoinositol when resolved by paper chromatography in five solvent systems; solvent system A, $R_F = 0.26$ (glucose = 0.44); B, $R_F = 0.23$ (glucose = 0.51); C, $R_F = 0.12$ (glucose = 0.31); D, $R_F = 0.34$ (glucose = 0.47) and E, $R_F = 0.11$ (glucose = 0.22). The acetate derivatives formed from the isolated compound (specific activity, $0.168 \mu\text{Ci}/\mu\text{mol}$, S.E. = 0.007, three replicates) had the same retention time during elution from the gas liquid chromatograph column as the acetate derivative prepared from authentic $[\text{U-}^{14}\text{C}]$ myoinositol and the specific activity of the compound remained constant after each of three re-crystallizations ($0.166 \mu\text{Ci}/\mu\text{mol}$, S.E. ± 0.014 ; $0.132 \mu\text{Ci}/\mu\text{mol} \pm 0.004$; $0.137 \mu\text{Ci}/\mu\text{mol} \pm 0.003$; $0.135 \mu\text{Ci}/\mu\text{mol} \pm 0.002$, three replicates).

Metabolism of intracellular inositol by testicular spermatozoa

When the intracellular metabolites formed from labelled glucose were extracted from testicular spermatozoa before and after re-incubation for 5 hr with no added substrate, the total radioactivity had decreased by about 25% ($14,000 \pm 1500$ S.E. counts/min/ 10^7 cells, versus $10,800 \pm 980$ counts/min/ 10^7 cells, $P < 0.05$, four replicates), whereas the concentration of inositol decreased by some 55 to 64% (6700 ± 250 counts/min/ 10^7 cells versus 2690 ± 132 counts/min/ 10^7 cells, $P < 0.01$). The radioactivity in the centre well accounted almost entirely for the loss of the intracellular radioactivity. However, at the same time, the radioactivity of at least two unidentified intracellular metabolites had greatly increased while that of others remained almost constant.

The total oxygen uptake of such spermatozoa was $19.6 \pm 2.29 \mu\text{l}/10^8$ cells. Since their respiration was almost identical to that of washed testicular spermatozoa which had not been pre-incubated with glucose ($19.2 \pm 1.12 \mu\text{l}/10^8$ cells), the carbon dioxide accumulating must have arisen from endogenous sources. By contrast, the addition of glucose stimulated the oxygen uptake of testicular spermatozoa by about 60% ($19.2 \pm 1.12 \mu\text{l}/10^8$ cells versus $30.6 \pm 1.24 \mu\text{l}/10^8$ cells).

Metabolism of extracellular inositol

Myoinositol added to calcium-free Krebs-Ringer phosphate diluent was ineffective in stimulating the respiration of testicular or ejaculated spermatozoa, although both types of spermatozoa converted small amounts of the substrate to

carbon dioxide (Table 2). Under these conditions of incubation, substrate carbon accumulated in testicular but not in ejaculated spermatozoa or the incubation medium from both cell types. Rete testis fluid increased the oxygen consumption of washed testicular spermatozoa by about 45% and that of washed ejaculated spermatozoa by 140%. However, while the fluid was ineffective in stimulating the oxidation of inositol by ejaculated spermatozoa, it increased the accumulation of intracellular substrate carbon in both testicular and ejaculated spermatozoa.

DISCUSSION

The present results constitute clear evidence of inositol synthesis in testicular spermatozoa and support the suggestion of Eisenberg & Bolden (1964) that spermatozoa may be a site of inositol synthesis from glucose. However, the activity of testicular spermatozoa is unlikely to account entirely for the high inositol concentration in rete testis fluid (Setchell *et al.*, 1968) since inositol concentrations considerably in excess of blood plasma have been recorded when sperm numbers were less than 0.01% of normal although above 10^6 spermatozoa/ml, the inositol concentration was positively correlated with sperm numbers (Voglmayr, Hinks, White & Setchell, 1970; Setchell, Voglmayr & Hinks, 1971).

Despite differences in sperm concentration and composition of the incubation medium used in the present investigation, the testicular spermatozoa displayed their characteristic pattern of glucose dissimilation. Confirming earlier results, carbon dioxide accounted for about 25%, lactic acid for 45% and amino acids for 5% of the glucose utilized (Voglmayr *et al.*, 1967). Since a smaller percentage of the oxygen taken up by testicular spermatozoa is required for the oxidation of the utilized glucose, these cells presumably oxidize relatively more endogenous substrate than ejaculated spermatozoa, which have been shown to oxidize only negligible amounts of endogenous material in the presence of hexoses (Wales & O'Shea, 1966).

The absence of inositol in ejaculated ram spermatozoa confirms previous observations (Mann, 1964) and the metabolites accumulating in these cells presumably constitute intermediates of the Embden-Meyerhof glycolytic pathway and the Krebs' cycle. Thus, O'Shea & Voglmayr (1970) found that some 30 to 40% of the intracellular substrate carbon accumulated by ejaculated spermatozoa from glucose was acetate; some lactate was also present, whereas in testicular spermatozoa only small amounts of these metabolites were detectable.

The endogenous inositol utilized by testicular spermatozoa in the absence of added hexoses may be involved in resynthesis of inositol-containing phospholipids which are utilized by the spermatozoa during their period of maturation in the epididymis (Scott *et al.*, 1967) presumably after hydrolysis and oxidation of the fatty acid moiety (Mills & Scott, 1969). Alternatively, the inositol occurring in testicular spermatozoa and in the fluids of the male genital tract may serve as a 'reserve carbohydrate' for hexoses (Mann, 1964). Fischer (1945) has emphasized the close structural similarity between inositol and glucose and

when labelled inositol is injected into rats it is, at least partially, converted to labelled glucose (Posternak, Schopfer & Reymond, 1955).

The small but significant amount of extracellular inositol oxidized by ejaculated spermatozoa confirms the results of O'Shea & Wales (1966) but the present results with testicular spermatozoa are in contrast to those of Setchell *et al.* (1968) who found no evidence of the oxidation of the cyclitol by testicular spermatozoa incubated in Krebs-Ringer bicarbonate. It is possible that the phosphate of the diluent used in the present investigation may stimulate the oxidation of the cyclitol by the testicular spermatozoa since phosphate has been shown to increase the oxidative metabolism of these cells in the presence of glucose (Voglmayr *et al.*, 1967).

The absence of exogenous metabolites in the reaction mixtures, containing inositol, suggests that the rate of accumulation of conversion products, if any, may be slow and just adequate to meet the demands of the Krebs' cycle. Since the addition of exogenous inositol failed to increase the oxygen consumption of the spermatozoa above control values, this substance presumably decreases the oxidation of endogenous substrates in the spermatozoa as, for instance, do hexoses under certain conditions of incubation (Scott, White & Anison, 1962; Voglmayr *et al.*, 1967).

It is uncertain which cellular components of testicular spermatozoa are concerned with the uptake of glucose for synthetic reactions. However, the cytoplasmic droplet may be involved since after incubating testicular spermatozoa with [3-³H]D-glucose, this structure appears to display a greater radioactivity than any other segment of the spermatozoon as judged by autoradiography (Voglmayr & White, unpublished). The observation that impairment of the uptake of glucose by testicular spermatozoa is associated with striking structural changes in the cytoplasmic droplet also supports this theory (Voglmayr, Setchell & White, 1971).

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