METABOLISM OF SPERMATOOZOA AND COMPOSITION OF FLUID COLLECTED FROM THE RETE TESTIS OF LIVING BULLS

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Summary. A technique for collecting spermatozoa and fluid directly from the testis of the living bull is described. Rete testis fluid was secreted at a mean rate of 12.0 ml/100 g of testis/day and spermatozoa were present at a mean concentration of 92×10^6 cells/ml. Chemical analyses have been made on rete testis fluid and its constituents compared with those of blood plasma from the same bulls. The fluid is iso-osmotic with blood plasma and contains less sodium, calcium, magnesium, inorganic phosphorus and protein but more potassium, chloride and glutamic acid. The concentration of urea and lactic acid was similar in rete testis fluid and blood plasma. Testosterone and androstenedione were found in the fluid of one mature bull at a concentration of 2.7 and 1.7 $\mu\text{g}/100$ ml of fluid, respectively.

High concentrations of phosphate (80 mM) depress the respiration and glycolysis of ejaculated spermatozoa but stimulate that of testicular spermatozoa. In low phosphate diluent (20 mM), testicular spermatozoa converted a higher percentage of the utilized glucose to carbon dioxide than ejaculated cells and, under all conditions of incubation, accumulated more lactic acid in the media than could have arisen from the glucose utilized. There was no evidence of pentose-shunt activity in either testicular or ejaculated spermatozoa.

INTRODUCTION

The epididymis is an important part of the excurrent duct system in which spermatozoa undergo extensive morphological and physiological changes known as 'maturation' (see Bishop & Walton, 1960; Bishop, 1961; Mann, 1964). In the bull, this requires an average of 11 days (Orgebin-Crist, 1961) and, in order to understand the process, fluid (Redenz, 1925; Salisbury & Cragle, 1956; Crabo & Gustafsson, 1964; Crabo, 1965; Gustafsson, 1966; Wales, Wallace & White, 1966) and spermatozoa (Redenz, 1933; Henle & Zittle, 1942; Lardy, Hansen & Phillips, 1945) collected from the testis and epididymis at slaughter have been studied. Recently, such studies have been

greatly facilitated by the development of a technique for collecting spermatozoa and fluid directly from the testis of living rams (Voglmayr, Waites & Setchell, 1966; Voglmayr, Scott, Setchell & Waites, 1967; Scott, Voglmayr & Setchell, 1967; Setchell, Hinks, Voglmayr & Scott, 1967; Murdoch & White, 1968). This paper describes an extension of the technique to the bull and, to our knowledge, this study represents the first evaluation of testicular secretion from this animal while still alive.

MATERIALS AND METHODS

Animals

Two Holstein bulls (1½ years old and weighing 450 and 500 kg) and one Ayrshire bull (3 years old, weighing 700 kg) were fed on 15 kg lucern hay per day. The animals were supported by a heavy duty canvas sling in a covered yard.

Collection of testicular secretion

The surgical procedure was similar to that described previously for the ram (Voglmayr *et al.*, 1967). General anaesthesia was induced by intravenous injection of 10% thiopentone sodium (10 mg/kg) and maintained with Fluothane (ICI) administered in a closed circuit system. Under aseptic precautions, the head of the epididymis was exposed through a 5-cm incision on the lateral surface of the scrotum and tunica vaginalis. The head of the epididymis was then carefully dissected from the tunica albuginea until the efferent ducts were located. Dissection was continued around the tubules until two fine ligatures could be passed behind these. Exerting tension on the tubules by means of one of the two ligatures, a cataract needle (5 × 2 mm, Heiss, Germany) was inserted through the tubules into the rete testis (Pl. 1, Fig. A). The incision was extended with dilators (Pl. 1, Fig. B) so that a fine silicone rubber catheter (Voglmayr *et al.*, 1967) with a stainless steel stillette in its lumen could be placed into the rete testis (Pl. 1, Fig. C). After securing the catheter above its cuff with the second ligature around all the efferent ducts, the stillette was withdrawn (Pl. 1, Fig. D). The catheter was brought to the exterior as described by Voglmayr *et al.* (1967). The testicular secretion was collected into a polyethylene receptacle attached to the anterior surface of the scrotum and was kept below 20° C by evaporative cooling.

Collection of ejaculated spermatozoa

Semen was collected either by an artificial vagina or by electro-ejaculation using the apparatus described by Martin & Rees (1962).

Preparation and incubation of sperm suspensions

Testicular spermatozoa, collected over a period of up to 12 hr, were separated from rete testis fluid by centrifugation at 400 g for 6 min. Ejaculated semen was diluted with one volume of diluent and centrifuged at 400 g for 8 min. After aspirating the supernatant, the spermatozoa were resuspended in diluent and centrifuged again. The supernatant was removed and the spermatozoa

PLATE 1

Sagittal section through the testis of a mature bull, demonstrating successive steps in the technique for cannulating the rete testis. Fig. A. Cataract needle inserted through the efferent tubules (et) into rete testis (r) following dissection of the head of epididymis (h) from the tunica albuginea; v, vascular cone, t, testicular parenchyma, m, mediastinum. Fig. B. Incision dilated, Fig. C. Catheter inserted into dilated incision until its cuff is well inside efferent tubules. Fig. D. Catheter secured above cuff (cu) around all efferent tubules with second ligature and brought to exterior from under the head of epididymis.

(Facing p. 450)

PLATE 2

Photomicrograph of a sperm suspension collected from the rete testis of the living bull by the catheter. Note prominent kinoplasmic droplets. (Phase contrast.)

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were resuspended in appropriate incubation media to a final concentration of approximately 10^8 cells/ml/flask. The spermatozoa were incubated in Warburg flasks (3- to 4-ml) at 37° C and shaken at a rate of 120 strokes/min. Duplicate incubations were made on spermatozoa from each of at least two collections per animal. Before incubation, the washed sperm suspensions were checked for bacterial contamination by phase contrast microscopy and the percentage of dead spermatozoa assessed by staining with nigrosin-eosin (Hancock, 1952).

Diluents and substrate

Spermatozoa were incubated in either a high phosphate diluent (80 mM) containing 36 mM-sodium chloride, 4 mM-potassium chloride and 2 mM-magnesium chloride or a low phosphate diluent (20 mM) of the same ionic composition, where sodium chloride was added at the expense of phosphate (pH 7.1). Radio-active D-glucose [$U-^{14}C$], [$1-^{14}C$] and [$6-^{14}C$] (Radio-chemical Centre, Amersham, Buckinghamshire) was checked for its purity by chromatographic procedures (see below) and added to the media to give a final concentration of 0.6 μCi /700 μg /flask.

Analytical procedures

Oxygen uptake was measured at 30-min intervals by the direct manometric technique of Umbreit, Burris & Stauffer (1959). Radio-active carbon dioxide was collected in the centre well by the method described by Buhler (1962). After incubation, the sperm suspensions were deproteinized by adding 0.5 vol. of 5% (w/v) $ZnSO_4 \cdot 7 H_2O$ and 0.5 vol. of 0.3 N-Ba(OH)₂. Glucose, lactic acid and amino acids in the neutral filtrates were assayed by chromatographic procedures (Freinkel, Cohen, Arky & Foster, 1965) and by enzymatic methods (Huggett & Nixon, 1957; Barker & Britton, 1957; Sowerby & Ottaway, 1966). Radio-activity was measured by an automatic liquid scintillation spectrometer (Packard Instrument Co., La Grange, Illinois). Sodium and potassium were measured by flame photometry and calcium and magnesium were estimated by atomic-absorption spectroscopy (Willis, 1965). Chloride was determined by the procedure of Zall, Fisher & Garner (1956) adapted for the auto analyser and urea by the method of Wilson (1966), using urease and phenol. Inorganic phosphorus was estimated by the procedure of Fiske & Subbarow (1925) after deproteinization with trichloroacetic acid. Protein was measured with Folin phenol by the method of Lowry, Rosebrough, Farr & Randall (1951). Testosterone and androstenedione were identified chromatographically in a Bush A type system according to their mobility and u.v. absorption (Bush, 1952).

Statistical methods

The statistical significance of the results has been determined by analysis of variance.

RESULTS

Secretion of spermatozoa and fluid from the testis

Abundant quantities of testicular fluid were obtained for up to 10 days and

there was little contamination with extraneous cells (Plate 2). The volume of the fluid secreted by the testis of a mature bull was about 40 ml per day (12.0 ml/100 g of testis/day; S.E. ± 1.1) during the first 4 to 5 days and then declined to about 60% of this volume by the 10th day.

The concentration of spermatozoa remained fairly constant throughout the collection period and the output of spermatozoa followed the same pattern as the volume of the rete testis fluid. Spermatozoa, all with prominent kinoplasmic droplets, were present at a concentration of 91.6×10^6 cells/ml (S.E. ± 7.05 ; three bulls) so that the daily production was 1.1×10^9 spermatozoa/100 g of testis (S.E. ± 0.1).

The initial pH of the fluid was 7.2 but rose to 8.5 after several hours. Almost all the spermatozoa were immotile but a few showed slow oscillating movements without progression. Storage for 3 days at 3° C did not produce any increase in motility.

TABLE 1
CONSTITUENTS AND PROPERTIES OF RETE TESTIS FLUID AND
BLOOD PLASMA COLLECTED FROM THE SAME BULLS

Constituent	Rete testis fluid	Blood plasma
Sodium	306.3 $\pm$ 3.00	318.1 $\pm$ 1.44
Potassium	35.3 $\pm$ 1.39	16.7 $\pm$ 0.44
Calcium	1.9 $\pm$ 0.33	7.9 $\pm$ 0.24
Magnesium	1.0 $\pm$ 0.03	2.1 $\pm$ 0.05
Chloride	433.6 $\pm$ 3.18	330.7 $\pm$ 5.13
Inorganic phosphorus	0.08 $\pm$ 0.02	7.1 $\pm$ 0.99
Urea	35.1 $\pm$ 1.24	36.1 $\pm$ 1.99
Protein	210.6 $\pm$ 22.13	8997.3 $\pm$ 237.0
Glucose	trace	81.3 $\pm$ 1.68
Lactic acid	6.6 $\pm$ 1.66	13.1 $\pm$ 3.6
Glutamic acid	19.5 $\pm$ 1.59	7.2 $\pm$ 0.62
Osmolality (m-osmole/kg)	280.8 $\pm$ 2.86	281.5 $\pm$ 1.79

Values (mg/100 ml) are the mean $\pm$ S.E. from at least three collections from each of three animals.

Composition of rete testis fluid and comparison with blood plasma

Table 1 shows the concentration of constituents in the rete testis fluid and blood plasma of three bulls. Rete testis fluid was iso-osmotic with the blood plasma and contained less sodium but about twice as much potassium. Calcium and magnesium were present in much lower concentrations than in plasma but the fluid contained about 31% more chloride and about three times more glutamic acid than blood plasma. There was very little inorganic phosphorus in the rete testis fluid, about 1.1% of plasma values and only traces of glucose were detectable. The concentration of urea was similar in rete testis fluid and blood plasma. Lactic acid was present in either higher or lower concentrations than in plasma where it varied considerably (range 3 to 44 mg/100 ml) depending on the excitation of the animal during blood collection. Rete testis fluid contained very little protein, about 2.3% of blood plasma content. The fluid of one animal (a 3-year-old Ayrshire bull) contained 2.7 μ g testosterone and

1.7 μg androstenedione/100 ml, whereas in the other two bulls these steroids were not detectable.

Comparison of the metabolism of testicular and ejaculated spermatozoa

Oxygen uptake. The oxygen uptake of testicular spermatozoa was always linear over 3 hr incubation and was higher in high phosphate diluent than in low phosphate diluent (Text-fig. 1). In low phosphate diluent, the oxygen

TEXT-FIG. 1. Oxygen uptake of washed testicular (●) and ejaculated (○) spermatozoa from the same bulls incubated in (a) low phosphate and (b) high phosphate diluent. For further details of incubation, see text.

TABLE 2
SUMMARY OF THE ANALYSIS OF VARIANCE
FOR THE FINAL OXYGEN UPTAKE IN
TEXT-FIG. 1

Source of variation	d.f.	Variance ratios
Spermatozoa	1	19.30**
Diluents	1	0.07
Interaction	1	11.00**
Replicates	10	3.04
Residual	30	13.50†

** $P < 0.01$; † Residual mean square.

uptake of ejaculated cells was higher than that of testicular spermatozoa during the first 2 hr of incubation but thereafter declined and by 3 hr it was similar for both types of spermatozoa. In high phosphate buffer, the initial rate of respiration was similar for testicular and ejaculated spermatozoa but the

oxygen uptake of the latter declined progressively with further incubation so that by 3 hr there was a striking difference between the two cell types.

Glucose breakdown. Table 3 gives the results of experiments on the breakdown of [U-¹⁴C]glucose by testicular and ejaculated spermatozoa. In a low phosphate diluent (20 mM), the uptake of glucose by testicular spermatozoa was less than that of ejaculated cells (84 versus 145 µg). However, high concentrations of phosphate stimulated glucose breakdown by testicular spermatozoa and depressed the activity of ejaculated cells so that in the presence of 80 mM-phosphate, glucose was utilized at about the same rate by testicular and ejaculated cells (116 versus 117 µg).

TABLE 3
METABOLISM OF [U-¹⁴C]GLUCOSE BY TESTICULAR AND EJACULATED BULL SPERMATOZOA

<i>Diluent</i>	<i>Spermatozoa</i>	<i>Glucose utilized</i>	<i>Glucose oxidized</i>	<i>[¹⁴C]lactic acid formed</i>	<i>Total lactic acid formed</i>
Low phosphate (20 mM)	Testicular	84.0	28.0 (33.3)	53.0 (63.0)	84.2
	Ejaculated	145.0	29.0 (20.0)	102.0 (70.3)	106.8
High phosphate (80 mM)	Testicular	116.0	29.0 (25.0)	80.0 (69.0)	115.0
	Ejaculated	117.0	27.0 (23.1)	81.0 (69.2)	86.3

Values (µg/10⁸ cells) are the mean of two duplicate incubations from each of three bulls. The glucose utilized equals initial minus final amount; the glucose oxidized has been calculated from ¹⁴CO₂ collected in KOH (20% w/v) in centre well; percentages are given in parentheses and relate to the glucose utilized.

TABLE 4
SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 3

<i>Source of variation</i>	<i>d.f.</i>	<i>Variance ratios</i>			
		<i>Glucose utilized</i>	<i>Glucose oxidized</i>		<i>Lactic acid</i>
			<i>(% glucose uptake)†</i>	<i>(µg/10⁸ cells)</i>	
Spermatozoa (A)	1	22.10**	15.50**	0.00	3.89
Diluents (B)	1	0.12	0.07	0.06	0.49
Assay (C)	1				11.80**
Interactions					
A × B	1	21.82**	10.10**	2.99	19.74**
A × C	1				6.34*
B × C	1				995.80**
Replicates	10	8.50**	6.00*	4.09**	10.80**
Residual	30	469.0	15.0	17.7	684.0‡

* $P < 0.05$; ** $P < 0.01$; † as angles (arcsin √percentage) corresponding to percentages; ‡ 71 degrees of freedom.

In the low phosphate diluent, testicular spermatozoa also oxidized a higher proportion of the glucose to carbon dioxide than ejaculated cells (33.3 versus 20%) with a correspondingly lower proportional accumulation of labelled lactic acid (63 versus 70.3%). In the high phosphate diluent, the proportions of glucose oxidized to carbon dioxide and the proportions accumulating as lactate were similar for both types of spermatozoa.

TABLE 5
METABOLISM OF $[1-^{14}\text{C}]$ - AND $[6-^{14}\text{C}]$ GLUCOSE BY TESTICULAR AND EJACULATED BULL SPERMATOZOA

Spermatozoa	Position of ^{14}C label	Glucose uptake*	$[^{14}\text{C}]$ carbon dioxide formed*	Specific yield†	$[^{14}\text{C}]$ lactic acid formed*	$\frac{1-^{14}\text{C}}{6-^{14}\text{C}}$
Testicular	1	558.3 ± 102.5	87.3 ± 9.1	0.180 ± 0.025	301.6 ± 52.0	1.01 ± 0.022
	6	451.6 ± 43.4	79.0 ± 7.8	0.183 ± 0.022	299.8 ± 52.3	
Ejaculated	1	551.0 ± 99.8	132.8 ± 10.0	0.283 ± 0.054	273.7 ± 28.3	0.923 ± 0.053
	6	460.0 ± 59.0	138.3 ± 8.3	0.313 ± 0.030	300.5 ± 34.3	

Values are the mean $\pm$ S.E. of at least two duplicate incubations from each of three bulls. The flasks contained approximately 10^8 cells in 1.0 ml of low phosphate diluent. The glucose used equals initial minus final amount.

* Counts/min/ 10^6 cells.

† As defined by Wood, Katz & Landau (1963).

The radio-activity of the carbon dioxide and lactic acid accounted for more than 90% of the glucose utilized. No other metabolites were detectable with ejaculated spermatozoa but with testicular spermatozoa, about 2% (low phosphate diluent) and 5% (high phosphate diluent) of the utilized glucose appeared on the chromatogram with a mobility similar to certain amino acids (i.e. aspartic and glutamic acid; $R_F = 0.08$ to 0.12) between the origin and the glucose ($R_F = 0.58$). Further, testicular spermatozoa produced more lactic acid than appeared as [^{14}C]lactic acid on the chromatogram and only 64 to 70% of the total lactic acid present could have arisen from the radio-active glucose. Only traces of lactic acid were detectable in the washed sperm suspension before incubation.

Hexose monophosphate activity. Testicular and ejaculated spermatozoa were incubated in low phosphate diluent with glucose labelled specifically in the C-1 and C-6 positions. The ratios of the yields of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]glucose to that from [$6\text{-}^{14}\text{C}$]glucose were 1.0 and 0.92 for testicular and ejaculated spermatozoa respectively and calculations of the specific yields of carbon dioxide, as defined by Wood, Katz & Landau (1963), showed that glucose dissimilation through the pentose cycle (hexose monophosphate shunt activity) was negligible in both types of spermatozoa (Table 5).

This was further substantiated by the similarity of the amounts of radio-active lactic acid accumulated by both cell types from C-1 and C-6 glucose.

DISCUSSION

This successful technique for cannulating the testis of the living bull should greatly facilitate studies of testicular spermatozoa and the fluid in which they are transported into the head of the epididymis. Not only does it make abundant quantities of testicular secretion available for several days, but it also reduces the risk of contamination by other cells and body fluids and the dangers of post-mortem changes inherent in studies with slaughterhouse material.

A comparison of the volume (about 40 ml) of fluid secreted daily by the testis of a mature bull with that obtained from the cannulated epididymis (Bennett & Rowson, 1963) substantiates the finding of Crabo (1965) that most of the fluid is resorbed by the epididymis. All the evidence suggests that this may take place in the first three segments of the bull's epididymis, resulting in a high concentration of spermatozoa (Nicander, 1958). The daily secretion of fluid and spermatozoa per 100 g of testis was about 62 and 75% respectively of that reported for Merino rams, although the total output per testis is of the same order in both species (Voglmayr *et al.*, 1966; Voglmayr & Mattner, 1968). The daily output of spermatozoa by each bull was comparable to the number of spermatozoa obtained by frequent ejaculation (Almquist, Hale & Amann, 1958) but was only half the sperm production of dairy bulls as calculated from gonadal sperm reserves and quantitative testicular histology (Amann & Almquist, 1962).

The composition of bull rete testis fluid was basically similar to that of the ram (see Setchell, Scott, Voglmayr & Waites, 1969). However, the fluid

collected from living bulls contained less potassium, calcium, lactic acid and protein but more urea and chloride than that obtained from animals at slaughter (Crabo, 1965; Wales *et al.*, 1966). The different concentration of most constituents in rete testis fluid and blood plasma strongly suggests the existence of the same blood–rete testis fluid barrier recently reported for the ram (Setchell, Voglmayr & Waites, 1969) and may have its site in the well-defined boundary tissue of the seminiferous tubules (Clermont, 1958; Lacy & Rotblatt, 1960; Gardner & Holyoke, 1964; Schmidt, 1964; Kagayama, Irisawa, Shirai & Matsushita, 1965; Ross & Long, 1966; Idänpään-Heikkilä, 1966; Ross, 1967). Certain characteristics of the fluid, particularly the high chloride concentration, indicate that rete testis fluid is a product of active secretion and not merely filtration.

Testosterone, when present, appeared in bull rete testis fluid at approximately the same concentration as in the fluid collected from living rams (Voglmayr *et al.*, 1966; White & Hudson, 1968) and the daily secretion, by way of the seminiferous tubules, from an average size testis was about 1% of the total testicular content (Lindner & Mann, 1960). Thus, the developing germ cells and testicular spermatozoa are exposed to relatively high concentrations of testosterone which may play an important rôle in the maintenance of spermatogenesis (Walsh, Cuyler & McCullagh, 1934). This high androgen environment extends into the head of the epididymis and may have important consequences for the survival of spermatozoa during their period of maturation (Moore, 1928; White & Hudson, 1968).

Glutamic acid, the principal free amino acid in ram rete testis fluid (Setchell *et al.*, 1967), was found in similar concentrations in the fluid collected from bulls. From these results, it can be calculated that secretion by the testis could account for most of the glutamic acid present in the bull ejaculate (Bhargava, Bishop & Work, 1959). Setchell *et al.* (1967) have demonstrated that glutamic acid does not pass through the limiting tissue of the seminiferous tubules but is synthesized from glucose. Preliminary observations also suggest an inverse relation between the glutamic acid and glucose concentration in rete testis fluid and a direct correlation with sperm numbers (Voglmayr & Setchell, unpublished observation). Thus, with decreasing sperm concentrations ($<20 \times 10^6$ cells/ml), less glutamic acid is detectable and at the same time significant amounts of glucose begin to appear in the fluid. At present, the significance of such high amino acid concentrations in rete testis fluid is difficult to assess; according to Setchell *et al.* (1967), they may create favourable conditions for nucleic acid synthesis within the seminiferous tubules since a number of amino acids have been shown to be involved in the synthesis of purine and pyrimidine bases (see Buchanan, 1960; Crosbie, 1960).

Testicular and ejaculated spermatozoa of the bull clearly differ in their response to the phosphate concentrations in the media. The present results confirm that high phosphate concentrations depress the respiration of ejaculated spermatozoa (see Salisbury & Lodge, 1962) and also their glycolysis, under certain conditions of incubation (Wales & Wallace, 1964). However, high phosphate concentrations stimulated the oxygen uptake of testicular spermatozoa and their utilization of glucose, which is in agreement with the

results obtained for the ram (Voglmayr *et al.*, 1967). In low phosphate diluent, testicular spermatozoa utilized less glucose than ejaculated cells and a greater percentage was converted to carbon dioxide. It seems likely that under these conditions, exogenous glucose can contribute a greater share of the carbon dioxide produced whereas, with high phosphate concentrations, more carbon dioxide is produced from concurrent endogenous metabolism. The rapid accumulation of lactic acid by bull testicular spermatozoa incubated in high phosphate diluent is in marked contrast to ram testicular spermatozoa which produce much less lactic acid from exogenous glucose under the same conditions (Voglmayr *et al.*, 1967) but form considerable amounts of amino acids, mainly glutamic acid (Voglmayr *et al.*, 1967). Since more lactic acid was formed by bull testicular spermatozoa than could have arisen from the glucose breakdown, there may be endogenous sources in the cell. The possibility that these may be reserve carbohydrates for hexoses such as glycogen or inositol (see Mann, 1964), which are broken down as spermatozoa pass through the epididymis, warrants further investigation.

The absence of an active pentose cycle in ejaculated bull spermatozoa confirms the work of Scott, White & Annison (1962) but our results with testicular spermatozoa are in contrast to those of Wu, McKenzie, Fang & Butts (1959) who found evidence of hexose monophosphate activity in spermatozoa collected from incisions into the testis parenchyma. Contamination of their preparations with testicular parenchyma may explain these conflicting results since this tissue has an active pentose cycle (Bloom, 1955; Field, Pastan, Herring & Johnson, 1960; Setchell & Hinks, 1967).

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