EFFECTS OF POTASSIUM PLUS MAGNESIUM AND OF AEROBIC OR ANAEROBIC CONDITIONS ON THE METABOLITES PRODUCED FROM FRUCTOSE BY WASHED RAM AND BULL SPERMATOZOA

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Abstract

The main metabolites from fructose were carbon dioxide, lactate, and acetate. Generally the effects of the addition of potassium (1 mM) plus magnesium (2 mM) were greater with 1×10^8 ram spermatozoa per millilitre than with 4×10^8 cells per millilitre.

Lactate, which accumulated mostly in the incubation medium, was lost from the cells during their separation by gradient centrifugation. Addition of ions to dilute suspensions of ram spermatozoa increased the extracellular lactate.

In contrast, acetate was not removed from the cell by the washing procedure and was mainly intracellular with aerobic conditions but was extracellular after anaerobic incubation. Addition of potassium and magnesium during aerobic incubation increased the proportion of acetate found within the cell and this increase was greater with the more dilute suspension of ram spermatozoa. More intracellular acetate accumulated under aerobic than under anaerobic conditions and relatively more was found in the cells from the more dilute suspensions. However, the total amount of substrate carbon inside the cells was unaltered by changes in the conditions of incubation.

Extracellular acetate was greater under anaerobic conditions. While the addition of potassium and magnesium had no effect with anaerobic incubation these ions decreased extracellular acetate under aerobic conditions. Again this effect was not as great with 4×10^8 spermatozoa per flask.

Washed ram spermatozoa also formed acetate from endogenous material, in the presence of added fructose.

Washed bull spermatozoa accumulated acetate from fructose but more was extracellular than intracellular, and the amount and site of accumulation of acetate were not affected by addition of ions or by anaerobic conditions. Only 15–20% of the intracellular pool of substrate carbon in bull spermatozoa was acetate. As with ram spermatozoa, most lactate was found in the medium, with very little being intracellular.

I. Introduction

Acetate is formed during incubation by both ram (Scott, Voglmayr, and Setchell 1967; O'Shea and Wales 1968; Wales and Humphries 1969; O'Shea 1970) and bull (Flipse and Almquist 1955; Graves, Lodge, and Salisbury 1966; Vera Cruz, Lodge, and Graves 1967) spermatozoa. Melrose and Terner (1953) and Terner (1959) showed that bull spermatozoa utilize pyruvate under anaerobic conditions by means of a dismutation to lactate, acetate, and carbon dioxide, although they did not measure acetate production. Potassium and magnesium increase the metabolic rate of spermatozoa (see Mann 1964) but an effect on acetate accumulation has only been observed with dilute cell suspensions (O'Shea 1970) and was not seen when the dilution rate varied (O'Shea and Wales 1968).

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Therefore, the interactions of sperm concentration, addition of ions, and presence or absence of oxygen on the metabolites produced by ram spermatozoa were examined. As the site of action of factors controlling spermatozoal metabolism must be intracellular an examination of intracellular metabolites was included. A similar experiment was carried out with bull semen.

II. Materials and Methods

(a) *General*

Semen, diluents, incubation in Warburg flasks, and analytical techniques have been described by O'Shea and Wales (1966). The basic diluent used for washing the spermatozoa and for incubation consisted of 20 mM mono- and disodium phosphate buffer (pH 7·0), 127 mM sodium chloride, 30 mg/100 ml penicillin, and 50 mg/100 ml streptomycin. In the first two experiments small Warburg flasks containing 1 ml of reaction mixture were used, while in the last experiment 3 ml were incubated in larger flasks.

The packed cell volume was obtained by centrifuging cold-shocked spermatozoa at 4200 *g* until a constant reading was obtained. Radioactivity was assayed by liquid scintillation techniques (Patterson and Greene 1965).

Data expressed as percentages were converted to angles before statistical analyses were carried out. Where the analyses of variance are not presented in the tables, the standard errors of the means calculated from them are given, together with the associated degrees of freedom, and the statistical significance of the results is quoted in the text.

(b) *Treatment of Spermatozoa after Incubation*

The reaction mixtures of the first two experiments were separated into their constituent spermatozoa and media by centrifuging the cells through a lactose rinse solution, consisting of 10·2 g/100 ml lactose and 20 mM fructose, as described previously (O'Shea 1970). In the last experiment polyvinylpyrrolidine (PVP) replaced lactose to reduce possible contaminants in the chemical tests for sugars. The cells from each flask were separated from the 3 ml of medium by centrifuging them through 8 ml of a rinse solution of 10 g/100 ml PVP and 154 mM sodium chloride into 0·5 ml of a solution of 15 g/100 ml PVP, 5% (v/v) formalin, and 154 mM sodium chloride in a tapered centrifuge tube. The supernatant was removed for storage, and the tube refilled with water. Then the water was removed along with a portion of the rinse solution. This procedure for washing the tubes was repeated three times.

The spermatozoal plugs were deproteinized with 2·5% perchloric acid (PCA), brought to pH 8 with potassium hydroxide, and the potassium perchlorate removed by centrifuging at 4°C. The resultant solutions were evaporated to dryness in a stream of air and the residue dissolved in water.

(c) *Isolation and Identification of Compounds*

The metobolites produced were separated by two methods. Firstly by paper chromatography carried out in a descending system using the following solvents:

Solvent A—n-butanol–acetic acid–water (4 : 1 : 5 v/v) (Lugg and Overell 1948; Hanes and Isherwood 1949).

Solvent B—n-propanol–ammonia (28%)–water (6 : 3 : 1 v/v) (Hanes and Isherwood 1949; Caldwell 1953).

Solvent C—ethanol–ammonia (7·6N)–water (8 : 1 : 1 v/v) (Block, Durrum, and Zweig 1958).

Solvent D—acetone–ammonia (7·5N)–n-butanol (7 : 1 : 2 v/v) (Block, Durrum, and Zweig 1958).

Solvent E—ethyl methyl ketone–glacial acetic acid–water saturated with boric acid at 22°C (9 : 1 : 1 v/v) (Rees and Reynolds 1958).

Solvent F—n-butanol–pyridine–morpholinium tetraborate (0·05M, pH 8·6) (7 : 5 : 2 v/v) (Carminatti *et al.* 1965).

Aliquots of the PCA extracts of the spermatozoal plugs were chromatographed using solvent A. The radioactive peaks obtained were extracted and rechromatographed with the other solvent systems. In the third experiment, material running with fructose on paper chromatography was tested for fructose with resorcinol (Mann 1948; White 1959) and diphenylamine-*p*-anisidine (Bailey 1962).

Secondly the technique of partition chromatography on silicic acid with hexane–butanol solvents (O'Shea and Wales 1968) was used to isolate acetic acid and lactic acid from aliquots of the supernatants, the rinse solutions, the formalin solutions just above the plugs, and the PCA extracts of the plugs. The materials in the radioactive peaks were recovered for further identification. Those tentatively identified as acetate were steam-volatile and ran with authentic acid when chromatographed in solvent C. In addition, in the third experiment the material in this peak was examined by gas–liquid chromatography and enzymatically assayed for acetate (Bergmeyer and Moellering 1966). The peaks tentatively identified as lactate ran with authentic lactate on paper chromatography with solvents A, B, and C. In the third experiment this material was tested enzymatically for lactate (Barker and Britton 1957).

III. Results

(*a*) *Metabolism of Ram Spermatozoa*

The interactions of the effects of sperm concentration, addition of ions, and gas phase were examined. Washed ram spermatozoa, at either 1 or 4×10^8 cells per millilitre, were incubated with fructose (20 mM) for 3 hr with either air or nitrogen as the gas phase, and with and without the inclusion of potassium (1 mM) plus magnesium (2 mM) ions in the incubation diluent. Mean results for four ejaculates are given in Tables 1–3.

Table 1

OXIDATIVE METABOLISM OF WASHED RAM SPERMATOZOA

Values are the means for four ejaculates expressed as μmoles per 10^8 spermatozoa over the 3 hr experimental period

No. of Cells per Flask	Addition of Potassium + Magnesium	Oxygen Uptake	Carbon Dioxide from Fructose
1×10^8	−	1·76	1·54
	+	2·42	2·24
4×10^8	−	2·24	1·98
	+	2·21	1·98
Standard error of the means		0·10	0·17
Degrees of freedom		9	9

There was a significant interaction between the effects of the addition of ions and of sperm concentration on respiration ($P < 0{\cdot}01$). Addition of potassium plus magnesium with 1×10^8 cells per millilitre increased the oxygen uptake, but with 4×10^8 cells per millilitre had no effect (Table 1). A similar tendency in respect of the oxidation of fructose was not statistically significant ($F = 4{\cdot}57$, d.f. $= 1 : 9$).

The mean results for the accumulation of metabolites and the associated analyses of variance are summarized in Table 2. There was more intracellular acetate under aerobic than under anaerobic conditions, and relatively more with the lower sperma-

Table 2

EFFECT OF THE ADDITION OF POTASSIUM (1 mM) PLUS MAGNESIUM (2 mM) ON THE ACCUMULATION OF METABOLITES FROM FRUCTOSE BY WASHED RAM SPERMATOZOA

Values are the means for four ejaculates expressed as μg-atoms of substrate carbon per 10^{10} spermatozoa over the 3 hr experimental period

Treatment	No. of Cells per Flask	Addition of Ions	Intracellular						Extracellular	
			Total	Acetate	Lactate	Peak 1	Peak 2	Peak 3	Acetate	Lactate
Aerobic	1×10^8	−	31·1	15·8	0·0	2·4	11·6	0·0	9·8	428
		+	29·4	14·0	0·1	1·7	10·9	0·4	0·9	639
	4×10^8	−	32·1	12·5	1·5	2·3	10·0	1·7	2·6	449
		+	32·4	15·3	2·7	2·1	10·4	0·1	0·6	508
Anaerobic	1×10^8	−	31·5	9·5	0·6	4·6	10·2	1·2	29·0	769
		+	25·3	9·1	0·3	3·1	8·6	0·0	46·9	1,133
	4×10^8	−	29·0	6·4	4·0	3·0	12·4	1·4	39·0	846
		+	26·9	6·0	1·9	2·0	13·3	0·1	36·9	850

Summary of the Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios						
		Substrate Carbon in Cell	Acetate in Cell	Lactate in Cell	Peak 1	Peak 2	Extracellular Acetate	Extracellular Lactate
Gas phase (A)	1	3·19	63·71***	3·58	14·87***	0·19	293·94***	112·22***
Number of cells (B)	1	0·21	6·08*	47·29***	3·23	1·66	0·90	4·52*
Addition of ions (C)	1	1·93	0·00	0·71	9·48**	0·06	0·33	18·44***
Interactions								
$A\times B$	1	0·51	1·66	0·53	9·55**	5·93*	0·90	0·41
$A\times C$	1	1·03	0·26	6·98*	1·95	0·01	10·97**	0·45
$B\times C$	1	0·80	1·94	0·26	0·57	1·01	2·70	11·92**
$A\times B\times C$	1	0·09	1·82	4·65*	0·00	0·14	11·14**	1·95
Ejaculate differences	3	23·25***	19·60***	0·75	6·68**	10·67***	5·86**	19·96***
Ejaculate interactions	21	*24·00*	*5·50*	*0·89*	*0·60*	*6·87*	*32·43*	*11,027*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

tozoal concentration. The three variables being investigated interacted in their effects on the accumulation of extracellular acetate. While there was much more acetate in the medium after anaerobic incubation, the effects of addition of potassium and magnesium varied. Under anaerobic conditions these ions had no effect with the higher cell concentration but increased extracellular acetate with dilute suspensions.

TABLE 3

EFFECT OF THE ADDITION OF POTASSIUM (1 mM) AND MAGNESIUM (2 mM) ON THE ACCUMULATION OF METABOLITES FROM FRUCTOSE BY WASHED RAM SPERMATOZOA

Values are the means for four ejaculates over the 3 hr experimental period

Treatment	Number of Cells per Flask	Addition of Ions	Percentage of Acetate Intracellular	Percentage of Intracellular Substrate Carbon Due to:		
				Acetate	Peak 1	Peak 2
Aerobic	1×10^8	−	62·4	49·8	7·0	35·5
		+	95·0	47·8	6·1	37·6
	4×10^8	−	83·1	39·4	6·8	32·3
		+	96·0	46·3	5·9	33·7
Anaerobic	1×10^8	−	23·3	30·5	14·5	32·4
		+	16·2	36·2	12·5	33·3
	4×10^8	−	12·9	20·7	10·4	41·2
		+	14·0	22·8	7·5	51·0

Summary of the Analyses of Variance (on transformed values)

Source of Variation	Degrees of Freedom	Variance Ratios: Percentage of Acetate Intracellular	Variance Ratios: Intracellular Carbon Due to:		
			Acetate	Peak 1	Peak 2
Gas phase (A)	1	307·08***	45·47***	20·41***	2·52
Number of cells (B)	1	0·25	10·30**	2·23	3·26
Addition of ions (C)	1	12·49**	1·65	1·12	1·48
Interactions					
$A \times B$	1	5·26*	0·35	3·74	8·48**
$A \times C$	1	17·63**	1·30	0·71	0·30
$B \times C$	1	1·21	0·17	0·43	0·39
$A \times B \times C$	1	5·09*	1·13	0·03	0·74
Ejaculate differences	3	1·72	6·26**	3·05	5·91**
Ejaculate interactions	21	*57·9*	*23·8*	*11·8*	*23·0*

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

Their addition under aerobic conditions decreased the amount of acetate in the medium with this decrease being greater in the more dilute suspensions. Although not tabulated, the changes in the total amounts of acetate accumulated ran parallel to those described for extracellular acetate.

There were only small amounts of lactate inside the cells and addition of potassium plus magnesium had no effect with dilute spermatozoal suspensions.

However, with 4×10^8 cells per millilitre these ions increased intracellular lactate under aerobic conditions and decreased this metabolite during anaerobic incubation. Extracellular lactate was increased by anaerobic conditions, but addition of the ions increased glycolysis only with the dilute cell suspensions.

When the PCA extracts of the spermatozoal plugs were chromatographed on paper with solvent A, four radioactive peaks were found. One of these was shown to be lactate (R_F 0·70) but the others were not positively identified.

Peak 1 was slow moving with most radioactivity at R_F 0·05–0·08. It mainly consisted of material with R_F 0·18–0·21 in solvent B, and R_F 0·06 in solvent C, similar to sugar phosphates. There were two other very minor components, one of which corresponded to inositol in chromatographic characteristics. More of the material of peak 1 was found in the absence of potassium and magnesium, and with 1×10^8 cells per millilitre more accumulated under anaerobic than under aerobic conditions.

Peak 2 had an R_F of 0·21–0·24 in solvent A, and mainly consisted of material running with fructose in solvents E and F but four other unidentified substances were present. Slightly more of peak 2 accumulated when the more concentrated sperm suspensions were incubated under nitrogen.

Peak 3, with an R_F of 0·37–0·42 in solvent A, was not always present.

When the acetate was calculated as percentage of acetate that was intracellular, or when the amounts of the metabolites were expressed as percentages of the total intracellular substrate carbon pool, other effects were seen (Table 3). A much smaller proportion of the acetate was intracellular under anaerobic conditions and this proportion was unaffected by addition of potassium and magnesium. During aerobic incubation addition of the ions increased the percentage of acetate found inside the cells, with the increase being greater at the lower spermatozoal concentration.

The gas phase used influenced the composition of the intracellular substrate carbon pool. Under aerobic conditions there was a greater proportion as acetate and a lesser proportion as peak 1. The percentage due to acetate was greater with dilute than with concentrated spermatozoal suspensions. There was an interaction of the effects of gas phase and of spermatozoal number such that most peak 2 was accumulated when 4×10^8 cells per millilitre were incubated under nitrogen.

A high percentage of the intracellular substrate carbon pool was extracted with PCA, and most of this carbon was accounted for by the chromatographic peaks (mean 89·4%, S.E. 5·6). There were no treatment effects both in regard to efficiency of PCA extraction and proportion of carbon accounted for.

(*b*) *Metabolism of Bull Spermatozoa*

Four ejaculates of bull semen were washed in the basic diluent and the cells ($1\cdot5$–$4\cdot5\times10^8$/flask) incubated for 3 hr in the presence and absence of potassium (1 mM) and magnesium (2 mM) chloride under aerobic and anaerobic conditions with fructose (20 mM) as substrate. It was found (Table 4) that addition of these ions slightly increased oxygen uptake ($t_3 = 2\cdot68$) and $^{14}CO_2$ production ($t_3 = 2\cdot50$) but did not affect the amount or site of accumulation of acetate. There was a greater accumulation of substrate carbon inside the cell under anaerobic than under aerobic

TABLE 4

EFFECT OF THE ADDITION OF POTASSIUM (1 mM) AND MAGNESIUM (2 mM) IONS ON THE METABOLISM OF WASHED BULL SPERMATOZOA

Values are the means for four ejaculates expressed per 10^{10} spermatozoa over the 3 hr experimental period

Treatment	Addition of Ions	Oxygen Uptake (μmoles)	Carbon Dioxide from Fructose (μmoles)	Intracellular Accumulation of Substrate Carbon (μg-atoms)					Extracellular Accumulation of Substrate Carbon (μg-atoms)	
				Total	Acetate	Lactate	Peak 1	Peak 2	Acetate	Lactate
Aerobic	−	141	127	17·4	3·0	0·7	3·0	7·6	10·3	298
	+	156	146	17·7	3·0	1·7	3·1	6·8	8·3	404
Anaerobic	−			24·3	4·3	1·4	3·6	10·9	5·8	690
	+			26·0	4·6	1·7	4·3	11·2	6·2	834
Standard error of the means		4·04	5·28	1·53	1·13	0·78	0·55	10·81	4·03	39·4
Degrees of freedom		3	3	9	9	9	9	9	9	9

conditions ($P < 0{\cdot}001$), and about half of this extra carbon was due to a concomitant rise in materials, termed peak 2, running in solvent A with R_F 0·20 ($P < 0{\cdot}01$). This peak was recovered and ran with fructose in solvent E. The material termed peak 1 remained close to the origin with solvent A and was a mixture of compounds. These peaks had the same chromatographic characteristics as peaks 1 and 2 obtained from ram spermatozoa.

Glycolysis, as measured by accumulation of extracellular lactate, was markedly increased by addition of ions ($P < 0{\cdot}05$) and by anaerobiosis ($P < 0{\cdot}001$).

When the distribution of acetate and the relative proportions of the constituents of the intracellular substrate carbon pool were examined (Table 5) no significant treatment effects were found.

TABLE 5

EFFECT OF POTASSIUM PLUS MAGNESIUM IONS ON THE ACCUMULATION OF METABOLITES BY WASHED BULL SPERMATOZOA

Values are the means for four ejaculates over the 3 hr experimental period

Treatment	Addition of Ions	Percentage of Acetate Intracellular	Percentage of Intracellular Substrate Carbon Due to:			
			Acetate	Lactate	Peak 1	Peak 2
Aerobic	−	48	15	4	17	45
	+	35	13	8	18	39
Anaerobic	−	48	20	5	15	44
	+	51	20	5	16	42

(c) *Identification of Metabolites*

To obtain further identification of the acetate and lactate fractions larger numbers of washed ram spermatozoa were incubated for 3 hr in the presence and absence of added potassium (1 mM) and magnesium (2 mM). In each flask some 25×10^8 cells were incubated with 60 μmoles of fructose and 11 μCi of [U-^{14}C]fructose. Duplicate flasks were run for two semen replicates. All of the PCA extract of the plug from one duplicate was chromatographed on silicic acid, and that from the other duplicate was chromatographed on paper with solvent A.

The materials from the plugs and supernatants running with lactate in both the silicic acid columns and in solvent A on paper were isolated and shown to react with lactate dehydrogenase.

The compound identified as acetate by elution from the silicic acid columns reacted as acetate in the enzyme system described in Section II, being converted to citrate. When subjected to gas–liquid chromatography 98% of the radioactivity was associated with acetate, although other volatile fatty acids were present. The mean specific radioactivity of the acetate was 23 ± 2 nCi per μmole. As the specific radioactivity of acetate formed from fructose was calculated to be 63 nCi per μmole some 64% of the acetate did not arise from fructose.

In this experiment the concentration of acetate in the PVP–saline solutions, both just above the plug ($1{\cdot}3 \pm 0{\cdot}15$ nCi/ml) and half way up the rinse solution

(1·5±0·15 nCi/ml) was much less than in either the supernatant (11·2±5·0 nCi/ml) or the plug (89±7 nCi/ml).

However, lactate concentration was similar in the plug (30±14 nCi/ml), in the PVP–formalin (35±12 nCi/ml), and in the PVP rinse solution (34±6 nCi/ml), but much greater in the supernatant (875±350 nCi/ml). Because of the greater volume of rinse solution there was a much larger absolute amount of lactate in the rinse solution than inside the cells.

Samples from peak 2 (R_F 0·21–0·24), obtained by chromatography of the PCA extract of the spermatozoal plugs in solvent A, were rechromatographed in solvents E and F. One half of each chromatogram strip was used to measure radioactivity, and the other for the fructose colour reactions described in Section II. A positive test for fructose was obtained, and the fructose peak coincided in outline with the main radioactive peak in both solvent E and solvent F.

IV. Discussion

The results of the last experiment were interpreted as confirming that the intracellular acetate was firmly bound (O'Shea 1970). The large amount of lactate in the rinse solution could be due to removal of medium contamination as suggested by the results of Wales and Humphries (1969) or to rapid loss of intracellular lactate. When measuring intracellular metabolites the background correction method of Wales and Humphries (1969) could be refined by using values for individual compounds in the formalin–saline solution to correct intracellular measurements and thus allow for different responses to washing the cells, as exemplified by lactate and acetate. The amount of acetate found in the spermatozoal plug is greater than that in the rinse solutions and does not need correcting to give a true measure of intracellular acetate. In contrast, the large amount of lactate in the rinse solutions means that uncorrected values for lactate in the spermatozoal plug do not accurately measure intracellular lactate.

The retention of a pool of "cold" acetate after incubation for 3 hr suggests that oxidation of plasmalogens (Hartree and Mann 1959, 1960*a*, 1960*b*, 1961) occurs in the presence of added fructose, although to a limited extent (O'Shea and Wales 1966). Such formation of metabolites from unlabelled endogenous substrates by spermatozoa would explain the reported accumulation of acids in some experiments (Vera Cruz, Lodge, and Graves 1967) but not in others where formation from labelled hexoses was measured (Wales and Humphries 1969).

That more than one mechanism is concerned in the formation of acetate from fructose by spermatozoa is shown by the different responses obtained under aerobic and anaerobic conditions. The amount and site of accumulation of acetate with aerobic incubation are dependent on the addition of potassium and magnesium as previously reported (O'Shea 1970). In contrast, most of the acetate found after anerobic incubation is extracellular and with dilute suspensions this extracellular acetate was increased by addition of these ions. Perhaps dismutation of pyruvate, which forms acetate anaerobically (Melrose and Terner 1953), does not result in "bound" acetate and is not important in the presence of oxygen plus fructose.

The results of the first experiment show that dilution rate of ram spermatozoa is critical in studying the effects of addition of potassium and magnesium on their respiration, accumulation of acetate, and formation of lactate.

Measurement of intracellular metabolites should be useful in examining control mechanisms as differences were seen with both ram and bull spermatozoa. Thus peak 2 was altered by incubation conditions with both species and the radioactivity in peak 1 from ram spermatozoa was less with addition of potassium and magnesium. Under normal conditions of incubation of spermatozoa hexose phosphates are not detected, although accumulation does occur in the presence of inhibitors or ATP (Hines and Smith 1963; Szepesi and Hopwood 1966; Flipse and Anderson 1969). However, Mann (1964) tabulates results showing that fructose in semen is mainly in the seminal plasma but the reverse is true for phosphohexose. The technique of gradient centrifugation thus provides a rapid preliminary concentration and separation of hexose phosphates from compounds in the medium.

The lack of effect of potassium plus magnesium on acetate accumulation by bull spermatozoa did not appear to be due to spermatozoal concentration, and the ions did increase formation of lactate. Differences in response to these ions by ram and bull spermatozoa have been reported by Murdoch and White (1966) but their report does not explain the present result.

Bull spermatozoa accumulate less intracellular acetate than ram spermatozoa, while their other intracellular metabolites appear similar. Under aerobic conditions most of the acetate accumulated by bull spermatozoa is extracellular. Again, comparing the results in Tables 2 and 4, spermatozoa of the bull accumulate, under nitrogen, much less extracellular acetate than do those of the ram. Investigation of the reasons for these differences would be interesting as acetate formation by ram spermatozoa is altered by cell maturation (O'Shea and Voglmayr 1970) and damage (O'Shea 1970).

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