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EFFECT OF IONS ON THE METABOLISM OF EJACULATED AND EPIDIDYMAL RAM SPERMATOZOA

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Summary. The effects of potassium, magnesium, calcium and phosphate ions on the metabolism of ejaculated and epididymal ram spermatozoa have been studied. Respiration and fructolysis were measured and isotopically labelled fructose was used to assess the contribution of fructose oxidation to total oxygen uptake.

Potassium and phosphate ions significantly increased both respiration and fructolysis; magnesium and calcium had much less influence and there were few significant interactions between the ions tested.

The most significant effect of washing spermatozoa free of seminal plasma was the reduction in the oxidation of substrates other than fructose. Washing also tended to accentuate the effects of other treatments.

There were differences between ejaculated and epididymal spermatozoa in the response of oxidative metabolism to potassium but, in general, the metabolic patterns of these cells were similar.

INTRODUCTION

The influence of the major biologically active inorganic ions on the viability and metabolism of spermatozoa has received considerable attention and the extensive literature on this topic has been reviewed recently by Salisbury & Lodge (1962). Interactions between ions have been reported to be significant in their effects on biological systems, and in the present studies factorially designed experiments have been used to study the ions in all combinations and to gauge the importance of possible interrelationships.

A number of parameters have been measured, with a view to localizing in broad terms the sites of action of these ions in the metabolic pathways of ram spermatozoa. In particular, with the availability of ^{14}C-labelled oxidizable substrates, it has been possible to investigate some of the factors influencing the endogenous respiration of washed spermatozoa in the presence of an exogenous substrate.

Since there are no reports of the effects of ions on the epididymal spermatozoa of the ram, the factors causing significant responses observed with ejaculated cells were subsequently examined in epididymal spermatozoa.

MATERIALS AND METHODS

Semen

Ejaculated semen was collected by electrical stimulation of the ram with a bipolar rectal probe as described by Blackshaw (1954). Epididymal spermatozoa were collected by gentle aspiration from the incised tubules of the cauda epididymis immediately after slaughter. Only samples of good initial motility were used, and care was taken to avoid sudden temperature changes during collection and handling.

Diluents

The basic diluent used in most experiments consisted of 20 mM veronal–HCl buffer (pH 7·2) and 134 mM sodium chloride. In one experiment veronal buffer was replaced by 20 mM trishydroxymethylaminomethane (tris) buffer pH 7·2. Where required potassium, magnesium and calcium were added to the diluents as chlorides and phosphate was added in the form of mono- and disodium phosphate buffer (pH 7·2). Isotonicity was maintained at 308 milliosmols per litre in all diluents by adjusting the sodium chloride content. The concentrations of the ions used in Experiment I were based partly on the levels reported for semen (White, 1958) and for both male and female genital tract secretions (Cragle, Salisbury & Muntz, 1958; Scott, Wales, Wallace & White, 1963; Olds & VanDemark, 1957).

Uniformally labelled fructose (D-U-^{14}C fructose from the Radiochemical Centre, Amersham, Bucks.) was added to the diluents to give a final concentration of 6 mM and a specific activity of 5·6 μmc/μmole in the incubation medium.

Washed spermatozoal suspensions

Samples of ejaculated spermatozoa were washed by diluting the semen with two volumes of basic diluent (or in some cases with tris-buffered saline) and centrifuging for 5 to 10 min at 300 ***g***. After aspirating the supernatant the spermatozoa were washed again and finally diluted to the original volume of semen with basic diluent. Epididymal samples, with a higher initial spermatozoal density, were diluted 1 in 10 for washing and, before incubation, the final washed suspensions were adjusted to give cell concentrations comparable with those of ejaculated samples.

The seminal plasma added to washed suspensions in the final experiment was obtained by centrifuging several pooled ejaculates at 0° C (500 ***g*** for 15 min). A portion of this plasma was dialysed for 24 hr in 'Visking' Cellulose dialysis tubing against two changes of 200 vol. basic diluent.

Incubation of spermatozoa

One volume of semen or washed spermatozoal suspension was added to 2 vol. of each diluent to give a concentration of 1 to 3×10^8 cells/ml in either 1 ml or 3 ml final volume. Incubation of the spermatozoa was at 37° C in Warburg flasks containing carbon dioxide-free 20% (w/v) KOH in the centre well. Oxygen utilization over a 2 to 3 hr period was measured with air as the gas phase, and a shaking rate of 120 strokes/min. The contribution of fructose

oxidation to total oxygen uptake was determined from the assay fo trapped $^{14}CO_2$ and the initial specific activity of the added fructose. Preliminary experiments had indicated that the methods used ensured quantitative recovery. Acidifying the spermatozoal suspension after incubation made no significant difference to the estimate of fructose oxidized ($t_{(5)} = 0{\cdot}6$; $0{\cdot}9 > P > 0{\cdot}8$) or to the contribution of endogenous substrate to the total respiration ($t_{(5)} = 1{\cdot}17$; $0{\cdot}3 > P > 0{\cdot}2$). Due to the possibility of other oxidizable substrates being present in the plasma of unwashed cells, the oxygen uptake not accounted for by fructose oxidation in unwashed cell suspensions has been called 'other oxygen uptake'. In washed cells where it is supposed that all exogenous substrates have been removed, the oxygen uptake not attributable to fructose oxidation has been referred to as endogenous oxygen uptake presumably arising from the breakdown of intracellular plasmalogen (Hartree & Mann, 1961). Aliquots of the spermatozoal suspensions prior to incubation and of the flask contents after incubation were deproteinized by the addition of 1 vol. 5 % w/v $ZnSO_4 \cdot 7H_2O$ and 1 vol. 0·3N $Ba(OH)_2$, and fructose and lactate were estimated in the neutral filtrates. At the end of each experiment total counts of spermatozoa were made in duplicate and all values were expressed as μmoles/10^8 cells over the experimental period. The motility of the spermatozoa in the Warburg flasks was checked at the completion of the experiments.

Analytical methods

Fructose was determined by the method of Mann (1948) as modified by White (1959), and lactate was determined by an enzymatic method, outlined by Barker & Britton (1957).

Assay of radioactivity

In the early experiments the contents of the centre well were quantitatively removed by rinsing with CO_2-free water and the $^{14}CO_2$ absorbed was precipitated as $Ba^{14}CO_3$ in the presence of 1 to 2 % ammonium chloride. The $Ba^{14}CO_3$ was collected by filtration on Whatman No. 542 paper as described by Annison & White (1961), and assayed for radioactivity with an end-window Geiger-Müller tube. The counts were corrected for self absorption by the method of Hendler (1959). In later experiments, metabolic carbon dioxide was assayed by liquid scintillation techniques as described by Buhler (1962) and counted in a Nuclear Chicago liquid scintillation spectrometer.

Statistical analysis

In order to scan, at the same time, as many combinations as possible, Experiment I was designed as a 2^6 factorial of thirty-two units (half replicate) in blocks of sixteen (Cochran & Cox, 1957). The six main factors were (A) potassium, (B) magnesium, (C) calcium, (D) phosphate, (E) washing, and (F) ejaculates as indicated in Table 1. Fractional replication was used since a fully replicated experiment requiring thirty-two Warburg flasks per ejaculate was impracticable. The defining contrast chosen was ABCDEF. It was considered that this would allow valid estimates of all main effects and their

first-order interactions, because these effects had fifth- and fourth-order interactions respectively as aliases and it was improbable that these high-order interactions would be large in comparison with the main effect.

All subsequent experiments on the effects of ions were fully replicated factorial experiments and their results were submitted to standard analyses of variance. In some experiments (Tables 4 and 5) the higher-order interactions have been used as error but in the smaller experiments pooled replicate interactions were used as error.

In order to equalize variances between the treatment groups, all results were transformed to logarithms before statistical analysis. The analyses are presented in summary form for the tables, giving only degrees of freedom (d.f.) and variance ratios for each source of variation. The residual (error) variance is given in italics at the base of each variance ratio column and asterisks are used to denote significant differences. All main effects and their first order interactions were isolated and tested for significance, but in the tables only the first-order interactions which were significant are presented separately. All non-significant interactions were combined and their pooled variance used to calculate a variance ratio.

In the final experiment, where several independent treatments were compared with controls, the standard error of the difference between each treatment and the control mean for each parameter were calculated from the interaction mean square of the analysis of variance of the logarithmically transformed data. The significance of the difference between means was then assessed by t-tests using the degrees of freedom associated with the interaction mean square above.

RESULTS

It is evident from the first experiment with washed and unwashed ejaculated spermatozoa (Table 1) that of the ions examined, potassium and phosphate had the most significant effects and there were few interactions between these and the other ions. Potassium stimulated both the aerobic fructolysis and the oxidation of fructose, but had no effect on the oxygen uptake attributable to oxidizable substrates other than fructose. The increases due to addition of potassium were almost exclusively in the washed spermatozoa, hence the significant interaction of potassium with washing in the analysis of variance. Phosphate, on the other hand, markedly stimulated all three energy-producing pathways studied. The increase in fructose oxidation was of the same order as that produced by addition of potassium, but fructolysis was approximately doubled by phosphate as compared with an increase of about 10% with potassium. The influence of phosphate on lactate accumulation was significantly greater in washed than unwashed cell suspensions. Calcium ions significantly depressed total oxygen uptake and fructose oxidation. These effects were more evident in the presence of phosphate than in its absence (*see* Text-fig. 1). At the concentration used the only significant effect of magnesium was to increase the oxidation of fructose by washed cells.

As well as accentuating some of the above effects, washing itself caused substantial changes. The contribution to oxygen utilization by oxidizable sub-

strates other than fructose was halved, while the oxidation of fructose was increased by 50 %. Significant differences between ejaculates were only evident in the oxidation of fructose and these differences were less in washed than

TABLE 1

MEAN EFFECTS OF IONS AND WASHING ON THE AEROBIC METABOLISM OF EJACULATED RAM SPERMATOZOA

Factor	*Level*	*Oxidative metabolism*			*Fructolysis*	
		Total O_2 uptake	*Fructose oxidation*	*Other O_2 uptake*	*Fructose utilization*	*Lactate accumulation*
A Potassium	0	1·59	0·175	0·54	0·99	1·79
	15 mM	1·74	0·201	0·54	1·15	2·00
B Magnesium	0	1·70	0·190	0·56	1·06	1·93
	5 mM	1·64	0·187	0·52	1·08	1·85
C Calcium	0	1·72	0·194	0·56	1·11	1·96
	1 mM	1·61	0·182	0·52	1·03	1·80
D Phosphate	0	1·55	0·177	0·49	0·76	1·18
	20 mM	1·78	0·200	0·59	1·38	2·59
E Washing	−	1·61	0·141	0·76	1·02	2·21
	+	1·73	0·235	0·32	1·12	1·56
F Ejaculates	1	1·73	0·202	0·52	1·11	1·84
	2	1·60	0·175	0·55	1·03	1·93

Summary of the analyses of variance

Source of variation	*d.f.*	*Variance ratios*				
		Total O_2 uptake	*Fructose oxidation*	*Other O_2 uptake*	*Fructose utilization*	*Lactate accumulation*
A Potassium	1	16·5**	21·6**	0·1	10·6**	8·6*
B Magnesium	1	2·1	2·2	2·4	0·0	0·2
C Calcium	1	7·9*	6·9*	1·5	1·1	1·5
D Phosphate	1	40·3**	23·6**	4·2	195·9**	407·2**
E Washing	1	10·2**	53·1**	94·1**	4·3	123·0**
F Ejaculates	1	17·0**	88·7**	0·0	3·2	3·6
First order interactions						
A × E	1	24·8**	27·4**	0·0	4·6	15·9**
B × E	1	0·5	6·3*	4·8	0·4	1·6
C × D	1	4·5	12·2**	0·0	8·9*	3·1
D × E	1	0·6	0·2	0·8	0·5	28·0**
E × F	1	19·9**	124·4**	1·6	19·4**	9·3*
Other	10	1·6	2·2	1·5	1·0	1·0
Residual	10	*7*	*8*	*155*	*27*	*26*

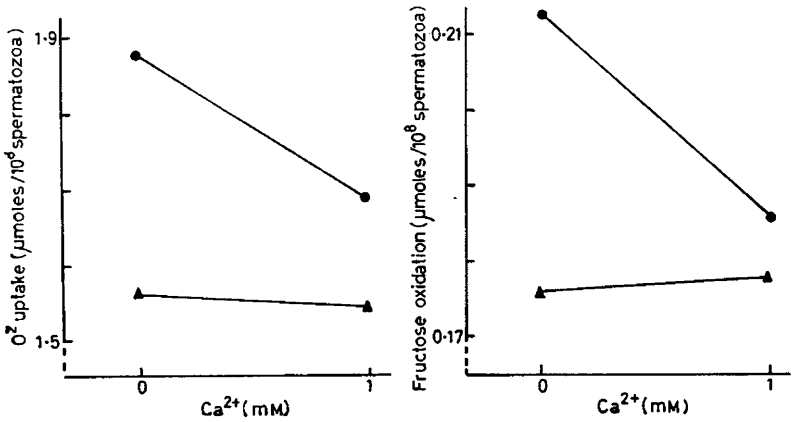
* $P < 0·05$; ** $P < 0·01$.

Values are expressed as μmoles/10^8 cells.

unwashed cells. In general, both ejaculates gave the same response to the other treatments studied.

Potassium and phosphate, which had the most significant effects on ejaculated spermatozoa (Table 1), were tested at the same concentrations on washed

epididymal spermatozoa. They were studied in factorial combination under both aerobic and anaerobic conditions, and the results, with summaries of the analyses of variance, are shown in Tables 2 and 3. Phosphate had similar



TEXT-FIG. 1. The interaction of calcium (1 mM) and phosphate (20 mM) ions on total oxygen uptake and fructose oxidation by ejaculated spermatozoa. ▲, 0 mM phosphate; ●, 20 mM phosphate.

TABLE 2

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE OXIDATIVE METABOLISM OF WASHED EPIDIDYMAL RAM SPERMATOZOA IN VERONAL-BUFFERED DILUENTS

Potassium (mM)	*Phosphate* (mM)	*Oxygen utilization*		*Fructose oxidation*
		Total	*Endogenous*	
0	0	1·32	0·158	0·202
0	20	2·12	0·618	0·251
15	0	1·70	0·397	0·218
15	20	2·19	0·658	0·255

Summary of the analyses of variance

Source of variation	*d.f.*	*Variance ratios*		
		Total O_2	*Endogenous* O_2	*Fructose oxidation*
A Potassium	1	6·6*	195·4**	0·9
B Phosphate	1	52·2**	707·5**	17·9**
Interaction A × B	1	4·2	158·2**	0·4
C Replicates	2	2·0	92·3**	0·4
Residual	6	*12*	*69*	*11*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and are the means for three animals.

effects on epididymal spermatozoa as on ejaculated cells but this was not true of potassium. Although the addition of potassium slightly increased the fructolysis and fructose oxidation of epididymal spermatozoa, the effects were not

significant. Endogenous oxygen uptake on the other hand was appreciably increased. However, there was a significant potassium × phosphate interaction and the effect of potassium was relatively less in combination with phosphate ions (*see* Text-fig. 2). There was no significant difference between the effects of

TABLE 3

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE AEROBIC AND ANAEROBIC FRUCTOLYSIS OF WASHED EPIDIDYMAL RAM SPERMATOZOA IN VERONAL-BUFFERED DILUENTS

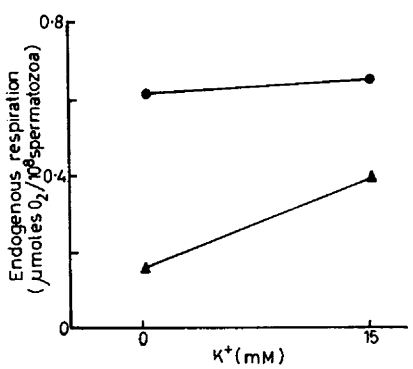
Potassium (mM)	*Phosphate* (mM)	*Fructose utilization*		*Lactate accumulation*	
		Anaerobic	*Aerobic*	*Anaerobic*	*Aerobic*
0	0	0·72	0·47	1·15	1·08
0	20	2·03	1·65	4·06	3·80
15	0	0·60	0·56	1·24	0·90
15	20	2·20	1·74	4·38	3·66

Summary of the analyses of variance

Source of variation	*d.f.*	*Variance ratios*	
		Fructose utilization	*Lactate accumulation*
A Anaerobic versus aerobic	1	2·1	9·9**
B Potassium	1	0·2	0·2
C Phosphate	1	9·4**	805·7**
Interactions	4	0·9	1·5
D Replicates	2	2·5	39·2**
Residual	14	*19*	*24*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and are the means for three animals.



TEXT-FIG. 2. The interaction of potassium (15 mM) and phosphate (20 mM) ions on the endogenous respiration of washed epididymal spermatozoa. ▲, 0 mM phosphate; ●, 20 mM phosphate.

aerobic and anaerobic conditions on fructose utilization and the significant difference in lactate accumulation between aerobic and anaerobic conditions was accounted for as fructose oxidized.

To study further the effects of potassium and phosphate on ejaculated spermatozoa under different conditions, samples of ejaculated semen were washed in veronal-buffered and tris-buffered basic diluent and incubated under aerobic and anaerobic conditions. The results, with summaries of the analyses of variance, are shown in Tables 4 and 5. Washed ejaculated spermatozoa exhibited greater oxidative capacity in tris-buffered than in veronal-buffered

TABLE 4

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE OXIDATIVE METABOLISM OF WASHED EJACULATED RAM SPERMATOZOA IN TRIS- OR VERONAL-BUFFERED DILUENTS

Buffer	*Potassium* (mM)	*Phosphate* (mM)	*Oxygen utilization*		*Fructose oxidation*
			Total	*Endogenous*	
Tris	0	0	1·695	0·461	0·211
	15	0	1·697	0·332	0·227
	0	20	2·045	0·711	0·222
	15	20	1·916	0·601	0·219
Mean			1·838	0·529	0·220
Veronal	0	0	1·121	0·296	0·143
	15	0	1·212	0·246	0·166
	0	20	1·392	0·447	0·169
	15	20	1·465	0·350	0·191
Mean			1·320	0·338	0·167

Summary of the analyses of variance

Source of variation	*d.f.*	*Variance ratios*		
		Total O_2	*Endogenous* O_2	*Fructose oxidation*
A Buffers	1	106·4**	27·6**	32·4**
B Potassium	1	0·0	15·5**	4·0
C Phosphate	1	32·6**	59·1**	1·3
D Ejaculates	2	235·5**	70·3**	81·8**
First order interactions				
A × D	2	5·4*	4·8*	3·3
C × D	2	1·6	16·7**	2·9
Other	5	1·2	1·0	1·4
Higher order interations (error)	9	*10*	*72*	*22*

* $P<0·05$; ** $P<0·01$.

Values are μmoles/10^8 cells and are the means for three ejaculates.

diluents (Table 4) though the magnitude of the response differed significantly between ejaculates. Furthermore, two additional ejaculates tested at high spermatozoal density also showed a similar difference in respiration between buffers. The significantly greater fructose utilization (Table 5) in tris- than in veronal-buffered diluents was accounted for by the increase in fructose oxida-

tion, and there was no appreciable difference in lactate accumulation between the two buffer types. Fructose utilization was slightly greater and lactate accumulation somewhat less under aerobic conditions, the differences being largely accounted for as fructose oxidized. While not affecting total oxygen

TABLE 5

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE AEROBIC AND ANAEROBIC FRUCTOLYSIS OF WASHED EJACULATED RAM SPERMATOZOA IN TRIS- AND VERONAL-BUFFERED DILUENTS

Buffer	*Potassium* (mM)	*Phosphate* (mM)	*Fructose utilization*		*Lactate accumulation*	
			Anaerobic	*Aerobic*	*Anaerobic*	*Aerobic*
Tris	0	0	0·25	0·51	0·44	0·33
	15	0	0·29	0·59	0·58	0·41
	0	20	1·00	0·99	1·84	1·21
	15	20	1·00	0·99	1·85	1·14
Mean			0·64	0·77	1·18	0·77
Veronal	0	0	0·09	0·37	0·40	0·25
	15	0	0·22	0·49	0·50	0·33
	0	20	0·93	0·93	1·59	1·45
	15	20	0·95	0·98	1·70	1·44
Mean			0·55	0·69	1·05	0·87

Summary of the analyses of variance

Source of variation	*d.f.*	*Variance ratios*	
		Fructose utilization	*Lactate accumulation*
A N_2 versus air	1	26·6**	45·0**
B Buffers	1	8·9**	2·7
C Potassium	1	5·6*	6·0*
D Phosphate	1	172·0**	713·8**
E Ejaculates	2	5·8**	62·0**
First order interactions			
A × D	1	26·2**	1·0
B × D	1	7·9**	10·7**
B × E	2	0·0	3·6*
C × D	1	4·6*	5·0*
Other	9	1·5	0·7
Higher order interactions (error)	27	*18*	*59*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and are the means for three ejaculates.

uptake, the addition of potassium increased the contribution of fructose oxidation at the expense of endogenous respiration. Potassium increased fructolysis but the effect was less in the presence of phosphate. Phosphate itself again significantly stimulated both oxidative and fructolytic pathways.

The influence of phosphate ion concentration on the metabolism of washed

ejaculated and epididymal spermatozoa was tested on veronal-buffered diluents with and without the addition of 3 mM potassium ions and the results, with summaries of the analyses of variance, are presented for the oxidative pathways in Table 6 and for the aerobic fructolysis in Table 7. Although the low level of potassium in the diluent had no effect on the oxidative metabolism of ejaculated spermatozoa, it significantly stimulated the endogenous respiration of epididymal cells. This increase was reflected in turn in the total oxygen

TABLE 6

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE OXIDATIVE METABOLISM OF WASHED EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA

Potassium (mM)	*Phosphate* (mM)	*Ejaculated spermatozoa* (4)			*Epididymal spermatozoa* (3)		
		Total O_2 uptake	*Endogenous O_2 uptake*	*Fructose oxidation*	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*
0	0	1·52	0·404	0·187	1·37	0·158	0·202
0	20	1·91	0·614	0·217	2·12	0·616	0·251
0	60	1·93	0·732	0·200	1·59	0·297	0·216
3	0	1·49	0·418	0·179	1·73	0·497	0·205
3	20	1·91	0·612	0·217	1.90	0·464	0·239
3	60	2·07	0·826	0·208	2·20	0·833	0·228

Summary of the analyses of variance

Source of variation	*Variance ratios*							
	Ejaculated spermatozoa				*Epididymal spermatozoa*			
	d.f.	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*	*d.f.*	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*
A Potassium	1	0·8	0·0	0·4	1	5·8*	7·2*	0·5
B Phosphate								
linear (L)	1	86·5**	22·7**	2·6	1	6·7*	8·0*	31·4**
quadratic (Q)	1	15·2**	0·0	5·9*	1	7·3*	2·3	114·5**
Interaction A × B								
L × L	1	2·9	0·2	0·8	1	3·5	0·9	1·7
L × Q	1	0·4	0·0	0·0	1	9·2*	10·3**	8·6*
Replicates	3	94·8**	10·2**	18·6**	2	0·5	1·4	4·1
Residual	15	*6*	*172*	*30*	10	*30*	*296*	*139*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and the number of replications is given in parentheses.

utilization, but fructose oxidation by epididymal spermatozoa was not increased by the addition of potassium ions. In both ejaculated and epididymal spermatozoa there was a slight but significant tendency for potassium to increase fructolysis, and the effect was not influenced by the phosphate ion concentration in the diluent. Phosphate markedly stimulated respiration and fructolysis for both ejaculated and epididymal cells. The effects of 60 mM phosphate were similar to those of 20 mM phosphate except with epididymal spermatozoa for

which high phosphate ion concentration was more effective in stimulating oxidative metabolism in the presence of potassium than in its absence.

The effects of high levels of potassium (40 mM) on the metabolism of washed ejaculated and epididymal spermatozoa, with and without the addition of 20 mM phosphate, are shown in Tables 8 and 9. The endogenous oxygen uptake of ejaculated cells was significantly increased by potassium in the absence of phosphate but depressed by a combination of potassium and phosphate. Phosphate ions again were responsible for significant increases in both endogenous and exogenous respiration of epididymal spermatozoa. For ejaculated cells

TABLE 7

INFLUENCE OF POTASSIUM AND PHOSPHATE IONS ON THE AEROBIC FRUCTOLYSIS OF WASHED EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA

Potassium (mM)	*Phosphate* (mM)	*Ejaculated spermatozoa* (4)		*Epididymal spermatozoa* (3)	
		Fructose utilization	*Lactate accumulation*	*Fructose utilization*	*Lactate accumulation*
0	0	0·69	0·81	0·47	1·08
0	20	1·45	2·49	1·65	3·80
0	60	1·28	2·45	1·50	3·27
3	0	0·64	0·89	0·66	0·98
3	20	1·57	2·82	1·82	3·69
3	60	1·54	2·91	1·72	3·81

Summary of the analyses of variance

Source of variation	*Variance ratios*					
	Ejaculated spermatozoa			*Epididymal spermatozoa*		
	d.f.	*Fructose utilization*	*Lactate accumulation*	*d.f.*	*Fructose utilization*	*Lactate accumulation*
A Potassium	1	1·0	12·3**	1	10·1**	0·0
B Phosphate						
linear (L)	1	34·6**	391·9**	1	220·9**	186·1**
quadratic (Q)	1	15·6**	144·5**	1	93·6**	73·2**
Interaction A×B	2	0·1	0·18	2	1·3	1·2
C Replicates	3	7·5**	50·8**	2	1·8	6·1*
Residual	15	*15*	*25*	*10*	*30*	*47*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and the number of replications is given in parentheses.

the response was only significant in the total oxygen uptake. As before the fructolysis of both ejaculated and epididymal cells was significantly increased by the addition of 20 mM phosphate.

In the preceding experiments the effect of potassium on washed spermatozoa has been studied under a variety of conditions. To summarize these effects, the results in the presence of potassium have been calculated for each experiment as a percentage of those in the potassium-free diluents and these figures are

presented in Table 10. There were clearly some differences between ejaculated and epididymal spermatozoa in the metabolic pathways stimulated by this cation. Potassium (15 mM) stimulated fructose oxidation by ejaculated spermatozoa but did not increase the utilization of endogenous pathways. On the other hand the endogenous respiration of epididymal spermatozoa was markedly stimulated by all levels of potassium while fructose oxidation was not affected. There was also a greater effect of potassium on fructolysis by ejaculated than by epididymal spermatozoa.

TABLE 8

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE OXIDATIVE METABOLISM OF WASHED EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA IN VERONAL-BUFFERED DILUENTS

Potassium (mM)	*Phosphate* (mM)	*Ejaculated spermatozoa* (4)			*Epididymal spermatozoa* (3)		
		Total O_2 uptake	*Endogenous O_2 uptake*	*Fructose oxidation*	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*
0	0	1·52	0·404	0·187	1·37	0·158	0·202
40	0	1·56	0·494	0·177	1·74	0·455	0·215
0	20	1·91	0·614	0·217	2·12	0·616	0·251
40	20	1·79	0·358	0·240	2·14	0·635	0·250

Summary of the analyses of variance

Source of variation	*Variance ratios*							
	Ejaculated spermatozoa				*Epididymal spermatozoa*			
	d.f.	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*	*d.f.*	*Total O_2 uptake*	*Endogenous O_2 uptake*	*Fructose oxidation*
A Potassium	1	0·2	2·7	0·7	1	4·2	4·1	1·2
B Phosphate	1	14·6**	0·4	3·7	1	35·7**	16·6**	58·4**
Interaction A × B	1	0·8	12·2**	4·1	1	3·5	3·2	1·4
C Replicates	3	17·6**	17·6**	24·4**	2	3·9	4·1	2·2
Residual	9	*17*	*259*	*11*	6	*28*	*18*	*147*

** $P < 0·01$.

Values are expressed as μmoles/10^8 cells and the number of replications is given in parentheses.

The severe depression in the oxidation of compounds other than fructose caused by washing (Table 1) may be due to damage to the cells' endogenous respiration, or to removal of the seminal plasma. The presence of seminal plasma could affect the respiratory pathways either by the protective effect of proteins (or other macro-molecules) or by the provision of alternative oxidizable substrates. The final experiment was designed to test these possibilities and the results are presented in Table 11. Aliquots (0·3 ml) of washed spermatozoan suspension were added to 0·6 ml aliquots of basic diluent, 50 % (v/v) seminal plasma or 50 % (v/v) dialysed seminal plasma in basic diluent. For comparison similar aliquots of unwashed semen from the same ejaculates were diluted with

TABLE 9

EFFECTS OF POTASSIUM AND PHOSPHATE IONS ON THE AEROBIC FRUCTOLYSIS OF WASHED EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA

Potassium (mM)	*Phosphate* (mM)	*Ejaculated spermatozoa* (4)		*Epididymal spermatozoa* (3)	
		Fructose utilization	*Lactate accumulation*	*Fructose utilization*	*Lactate accumulation*
0	0	0·69	0·81	0·47	1·08
40	0	0·60	0·70	0·56	0·81
0	20	1·45	2·49	1·65	3·80
40	20	1·69	2·96	1·73	3·99

Summary of the analyses of variance

Source of variation	*Variance ratios*					
	Ejaculated spermatozoa			*Epididymal spermatozoa*		
	d.f.	*Fructose utilization*	*Lactate accumulation*	*d.f.*	*Fructose utilization*	*Lactate accumulation*
A Potassium	1	0·0	0·3	1	0·5	0·0
B Phosphate	1	39·5**	478·3**	1	142·5**	74·6**
Interaction A×B	1	0·3	7·0*	1	0·0	0·5
C Replicates	3	5·9*	27·1**	2	3·1	1·1
Residual	9	*18*	*27*	6	*29*	*35*

* $P<0·05$; ** $P<0·01$.

Values are expressed as μmoles/10^8 cells and the number of replications is given in parentheses.

TABLE 10

SUMMARY OF THE EFFECTS OF POTASSIUM ON THE METABOLISM OF WASHED RAM SPERMATOZOA

Potassium conc. (mM)	*No. observations*	*Total* O_2 *uptake*	*Fructose oxidation*	*Endogenous respiration*	*Fructose utilization*	*Lactate accumulation*
Ejaculated						
3	12	98	100	106	110	115
15	40	105	122	90	114	116
40	8	98	103	84	121	111
Epididymal						
3	9	115	100	168	116	104
15	6	113	104	136	105	101
40	6	111	103	141	108	98

Values are expressed as percentages of those obtained in the potassium-free diluents.

basic diluent. As in Table 1, the contribution to respiration of substrates other than fructose was significantly greater in unwashed than in washed cells in basic diluent. The addition of seminal plasma to the washed suspensions significantly increased the contribution of this component to total oxygen utilization, while the effect of seminal plasma, dialysed free of utilizable substrates, was not significant.

TABLE 11

INFLUENCE OF SEMINAL PLASMA ON THE METABOLISM OF WASHED EJACULATED RAM SPERMATOZOA

Treatment	*Oxidative metabolism*			*Aerobic fructolysis*	
	Total O_2 uptake	*Fructose oxidation*	*Other O_2 uptake*	*Fructose utilization*	*Lactate accumulation*
Washed + basic diluent (control)	3·13	0·442	0·48	2·54	5·77
Washed + seminal plasma	3·21	0·224**	1·87*	2·78	7·33
Washed + dialysed seminal plasma	3·67	0·499	0·68	2·47	5·15
Unwashed	4·03*	0·447	1·33**	3.28	6·95

* Significantly different from the washed control, $P < 0.05$.

** Significantly different from the washed control, $P < 0.01$.

Values are expressed as μmoles/10^8 cells and are the means for three ejaculates.

DISCUSSION

The effects of potassium, phosphate ions and washing on the metabolism of ram spermatozoa were found to be highly significant. Magnesium and calcium had much less effect, and, although there are many reports of the interrelationship of ions in biological systems, few significant interactions were demonstrated in the present studies.

These results support the reports of earlier workers that potassium increases the metabolism and motility of spermatozoa, especially after washing (Lardy & Phillips, 1943; Blackshaw, 1953a, b; White, 1953a, b, c; Wales & White, 1958a, b). There was not, however, any indication that potassium was detrimental to metabolism at high concentration as reported by Cragle & Salisbury (1959) for bull spermatozoa.

In the present series of experiments, potassium, where effective, generally only caused a 10 to 20 % increase in metabolism. Such an increase is close to the limits of error for estimating the various parameters and thus it is not surprising to find that changes of similar magnitude in different experiments may differ in their level of statistical significance depending on the error variance for that particular experiment. The response to added potassium in these experiments is much smaller than that reported by White (1953b) and this may well be due to the lower spermatozoal density and more extensive washing technique used by that author.

Phosphate ions at concentrations up to 60 mM significantly increased all three metabolic pathways studied in both ejaculated and epididymal spermatozoa, and in veronal- and tris-buffered diluents under both aerobic and anaerobic conditions. There would seem to be species differences in the effects of phosphate on the oxidative metabolism of spermatozoa since similar studies with bull spermatozoa (unpublished data) have confirmed the report by Lodge, Salisbury, Schmidt & Graves (1963) that even low levels of phosphate (2 to 7·5 mM) significantly depress the respiration of washed ejaculated spermatozoa of this species. Other (unpublished) studies of ours confirm the results of El Zayat & Van Tienhoven (1961), who found that the metabolism of fowl spermatozoa was not affected by phosphate.

In some cases the effects of phosphate and potassium in combination were less than would be expected from the sum of their separate effects. The observations of Stanbury & Mudge (1953) and Gamble (1957) that orthophosphate depresses mitochondrial potassium levels may have some bearing on the mechanism of the potassium and phosphate interaction in spermatozoa. On the other hand, there was a markedly elevated rate of metabolism in the presence of phosphate, and components other than potassium may be the rate-limiting factors under these conditions.

As previously noted (Lardy & Phillips, 1943; Blackshaw, 1953a) calcium depressed the metabolism of ram spermatozoa but did not modify the effects of potassium. The results presented here for magnesium would lead one to the same conclusion as Salisbury & Lodge (1962) that variations in the concentration of this ion within the physiological range have little effect on metabolism.

Veronal and tris buffers may not be entirely inert with respect to cellular function. Quastel & Wheatley (1933) have reported that veronal slightly inhibited the oxidation of glucose, pyruvate and lactate by brain tissue *in vitro*. Although it has been claimed that barbiturates uncouple oxidative phosphorylation (Brody, 1955), the results of Messer (1958) and of Aldridge & Parker (1960) suggest that not all cases of depressed metabolism in the presence of veronal are associated with any degree of uncoupling. On the other hand, tris can penetrate cell membranes (Omachi, Macey & Waldeck, 1961) and may act as an intracellular buffer. Amine buffers have also been shown to interfere with the uptake of potassium by bacteria (Macleod & Onofrey, 1954). With regard to spermatozoa, both tris and veronal have been found suitable as buffers for semen diluents (Blackshaw, 1953a; Wales & White, 1958c; Davis, Bratton & Foote, 1963). Tris has also been found to reduce the depressing effect of high concentrations of potassium during the chilling of ram spermatozoa from 30 to 0° C (O'Shea & Wales, 1964). In the present studies, tris-buffered diluents were slightly superior to those buffered with veronal. However, there was no indication that the presence of tris modified the effects of potassium at the levels used.

Early studies of the endogenous metabolism of spermatozoa could only be made after removing exogenous substrate by washing. Scott, White & Annison (1962), however, have demonstrated the value of ^{14}C-labelled compounds in assessing endogenous respiration of spermatozoa in the presence of exogenous substrate. By the use of this in these experiments, it was found that the greatest

effect of washing was the marked reduction in the oxidation of substrates other than fructose. The results of the final experiment indicate that the depression in 'other oxygen uptake' in the first experiment was due to the removal of dialysable seminal constituents. Recent observations (T. O'Shea and R. G. Wales, unpublished data) suggest that lactate is one of the potential substrates in seminal plasma which even in low concentrations would exert a 'sparing effect' on fructose oxidation.

There were some differences in the effects of potassium on the oxidative metabolisms of ejaculated and epididymal spermatozoa but, as concluded by White & Wales (1961), the metabolism of these two types of cell is generally similar.

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