THE OXIDATIVE UTILIZATION OF FRUCTOSE AND ACETATE BY WASHED RAM SPERMATOZOA IN THE PRESENCE OR ABSENCE OF POTASSIUM AND MAGNESIUM

By R. G. WALES* and T. O'SHEA*

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Summary

The oxidative utilization of fructose and acetate by washed ram spermatozoa has been studied during incubation in diluents containing varying levels of potassium and magnesium. Both ions stimulated metabolism, but the effects of a combination of the ions were less than would be expected from the sums of their separate effects. The effects of potassium were greater when acetate rather than fructose was the substrate.

A method is described for the rapid collection of spermatozoa for the measurement of intracellular constituents. There was a marked decrease in intracellular potassium on washing in potassium-free diluent. The presence of 1 mM potassium during incubation of the washed cells led to an uptake of this ion against a concentration gradient. Washing did not affect intracellular levels of magnesium.

The amount of substrate carbon within the spermatozoa was greater with fructose than with acetate as substrate. Both ions influenced the intracellular levels of substrate carbon and the possible sites of action of these ions is discussed.

I. INTRODUCTION

The effect of potassium and magnesium on the motility and metabolism of ram spermatozoa has received considerable attention (Lardy and Phillips 1943; Blackshaw 1953; White 1953*a*, 1953*b*; Dott and White 1964; Wallace and Wales 1964). Scott, White, and Annison (1962) have studied glucose and acetate metabolism by washed spermatozoa incubated in Ringer–bicarbonate, but a comparison of the oxidative utilization of various substrates by washed spermatozoa in the presence and absence of added potassium and magnesium has not been reported. Therefore, the influence of these ions on the metabolism of hexose and acetate by washed ram spermatozoa is investigated in the present paper. In addition to studying the utilization of these substrates, some investigations of the intracellular levels of potassium, magnesium, and substrate have been undertaken.

II. MATERIALS AND METHODS

(*a*) *General Methods*

Ram semen was collected by electrical stimulation and washed twice in a diluent consisting of 20 mM mono- and disodium phosphate buffer (pH 7·0), 125 mM sodium chloride, 30 mg% (w/v) penicillin, and 50 mg% (w/v) streptomycin as previously described (O'Shea and Wales 1965). For the incubations, sodium acetate,

* Department of Veterinary Physiology, University of Sydney.

D-fructose, D-glucose, and potassium and magnesium chloride, as required, were included in the diluent and isotonicity maintained by adjusting the sodium chloride content.

All incubations were carried out in double side-arm Warburg flasks of 15–25 ml volume. As described previously (O'Shea and Wales 1965), the washed spermatozoal suspensions were held in a side-arm during temperature equilibration and added to the main compartment, containing incubation diluent and isotope, after the manometers were sealed. The final volume in the incubation flask was 3·0 ml. To terminate metabolism in the first three experiments, 0·1 ml 6N H_2SO_4 was added to the suspensions from the other side-arm at the completion of incubation.

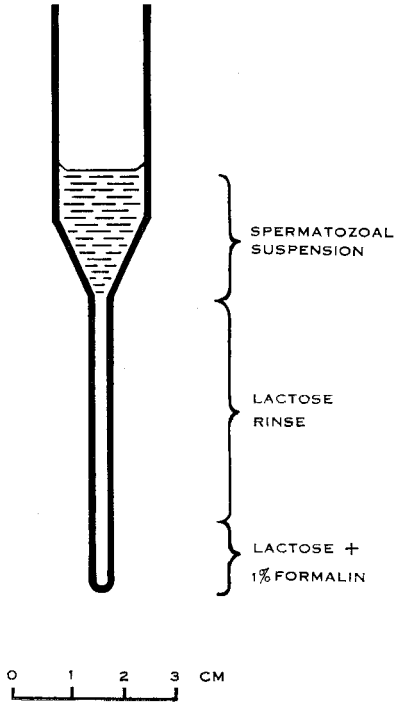

Fig. 1.—Centrifuge tube used to collect spermatozoa free of incubation medium.

The techniques used for the assay of radioactivity, the preparation of protein-free extracts of incubated suspensions, the assay of fructose, lactate and glucose, and the statistical analyses of the results have been described elsewhere (see O'Shea and Wales 1965). To allow ready comparison of the metabolism of acetate and hexose, substrate oxidation in the tables is expressed as its oxygen equivalent.

(*b*) *Measurement of Intracellular Constituents*

In order to measure the levels of potassium, magnesium, and substrate carbon in spermatozoa, a technique for the rapid recovery of spermatozoa free of diluent was developed from a method described by Lin and Geyer (1963) for the recovery of culture cells from radioactive media. The principle of the method is to collect spermatozoa free of medium by centrifuging them through an inert isotonic rinse solution.

A special centrifuge tube was constructed (see Fig. 1). The bottom 1 cm of the tube was filled with 11% (w/v) lactose solution containing 1% (v/v) formalin. The remaining narrow portion of the tube was filled with isotonic lactose. An aliquot of spermatozoal suspension (usually 0·9 ml) was then pipetted on the surface of the lactose solution and centrifuged for 15 min at 400 *g* to collect the cells as a plug.

Lactose (11% w/v) was chosen as the rinse as it had a high specific gravity (1·032 g/ml) and did not intermix with the overlying medium during centrifuging. Thus in a preliminary test, less than 0·06% of the label was recovered from the lactose layer after centrifuging 0·9 ml of medium containing 45 nanocuries of ^{14}C-labelled substrate. Formalin was included in the lactose at the base of the tube to stop further metabolism of substrates within the cell after separation from the incubation diluent. Preliminary tests indicated that approximately 1% formalin was required to inhibit the oxidation of fructose completely.

TABLE 1

LEVELS OF ISOTOPE, POTASSIUM, AND MAGNESIUM IN THE SUPERNATANT, LACTOSE LAYER, AND SPERMATOZOAL PLUG FOLLOWING INCUBATIONS OF WASHED SPERMATOZOA IN VARIOUS DILUENTS

Spermatozoal Cells Used and Layer Analysed	Isotope Level (counts/min/0·1 ml) in Cells Incubated		Potassium Level (mg/100 ml) in Cells Incubated		Magnesium Level (mg/100 ml) in Cells Incubated	
	with [^{14}C]Fructose	with [^{14}C]Acetate	without K^+	with K^+	without Mg^{2+}	with Mg^{2+}
Live cells						
No. of replicates	(16)	(16)	(8)	(8)	(16)	(16)
Supernatant	2,810	1,576	1·19	4·00	0·10	4·12
Lactose layer	172	88	0·10	0·42	0·11	0·33
Spermatozoal plug	6,647	13,116	69·33	126·95	16·65	18·85
Dead cells						
No. of replicates	(8)	(8)	(4)	(4)	(8)	(8)
Supernatant	3,070	2,750	2·57	5·85	0·29	4·12
Lactose layer	105	88	0·16	0·37	0·12	0·38
Spermatozoal plug	868	630	23·10	26·00	3·50	9·67

The whole spermatozoal plug and aliquots of the supernatant were removed for assay. Potassium and magnesium were measured by atomic absorption spectrophotometry. Radioactivity was assayed by liquid scintillation techniques using toluene–ethanol (2 : 1 v/v) containing 0·4% PPO* and 0·01% POPOP† as scintillator. After assay, intracellular concentrations of potassium, magnesium, and isotopic carbon were calculated using spermatocrit readings. Intracellular substrate carbon was then calculated from the isotopic data and the initial specific activity of the substrate. Thus it represents the contribution of substrate to the intracellular carbon pool at the termination of the experiment.

An indication of the effectiveness of the procedure can be gained from the results for analyses of supernatant, lactose layer, and spermatozoal plug given in Table 1. Correlation coefficients between supernatant, plug, and lactose were calculated for isotope, potassium, and magnesium. Those between supernatant and

* 2,5-Diphenyloxazole. † 1,4-Bis-2-(5-phenyloxazolyl)-benzene.

lactose were highly significant in each case [$r_{(47)} = 0{\cdot}71$, $P < 0{\cdot}01$; $r_{(23)} = 0{\cdot}65$, $P < 0{\cdot}01$; and $r_{(47)} = 0{\cdot}54$, $P < 0{\cdot}01$, respectively], while those between lactose and plug were not significant [$r_{(47)} = -0{\cdot}18$, $P > 0{\cdot}05$; $r_{(23)} = 0{\cdot}25$, $P > 0{\cdot}05$, and $r_{(45)} = 0{\cdot}14$, $P > 0{\cdot}05$, respectively].

TABLE 2

EFFECT OF POTASSIUM AND MAGNESIUM IONS ON THE METABOLISM OF WASHED RAM SPERMATOZOA WITH FRUCTOSE OR ACETATE AS EXOGENOUS SUBSTRATE

Values are expressed as μmoles/10^8 spermatozoa over the experimental period (3 hr) and, in each experiment, are the means for three ejaculates

Level of Ions Added		Fructose as Substrate				Acetate as Substrate	
K^+	Mg^{2+}	Total Oxygen Uptake	Oxygen Uptake from Substrate	Fructose Utilized	Lactate Accumulated	Total Oxygen Uptake	Oxygen Uptake from Substrate
				Experiment 1			
0	0	1·16	1·07	0·33	0·74	1·10	0·79
	3	1·63	1·33	0·72	1·20	1·44	1·09
Mean		1·40	1·20	0·53	0·97	1·27	0·94
5	0	1·84	1·44	1·11	1·55	1·91	1·64
	3	1·68	1·56	1·03	1·77	1·71	1·54
Mean		1·76	1·50	1·07	1·66	1·81	1·59
15	0	1·66	1·47	1·11	1·76	1·85	1·74
	3	1·72	1·43	1·24	1·92	1·76	1·71
Mean		1·69	1·45	1·18	1·84	1·81	1·73
S.E.M.*		—	—	0·09 (10)	0·16 (10)	—	—
				Experiment 2			
0	0	1·35	1·25	0·82	0·88	1·16	0·78
	0·5	1·55	1·28	1·01	1·05	1·43	1·05
	2	1·62	1·46	1·08	1·14	1·50	1·17
	8	1·75	1·48	1·05	1·19	1·56	1·15
Mean		1·57	1·36	0·99	1·06	1·41	1·04
5	0	1·74	1·62	1·32	1·75	2·10	1·67
	0·5	1·71	1·70	1·34	1·73	2·01	1·77
	2	1·73	1·47	1·36	1·76	1·98	1·77
	8	1·70	1·67	1·24	1·55	2·02	1·71
Mean		1·72	1·61	1·31	1·69	2·03	1·73
S.E.M.*		—	—	0·04 (14)	0·08 (14)	—	—

TABLE 2 (*Continued*)

Level of Ions Added		Fructose as Substrate				Acetate as Substrate	
K^+	Mg^{2+}	Total Oxygen Uptake	Oxygen Uptake from Substrate	Fructose Utilized	Lactate Accumulated	Total Oxygen Uptake	Oxygen Uptake from Substrate
				Experiment 3			
0	0	1·43	1·22	0·83	0·74	1·27	0·88
	2	1·99	1·58	1·21	1·31	1·60	1·14
Mean		1·71	1·40	1·02	1·02	1·44	1·01
0·2	0	1·62	1·50	0·92	1·31	1·56	1·00
	2	2·16	1·88	1·59	2·07	2·05	1·59
Mean		1·89	1·69	1·26	1·69	1·81	1·30
1·0	0	2·14	1·96	1·68	2·50	2·46	2·18
	2	2·34	2·12	1·67	2·35	2·53	2·24
Mean		2·24	2·04	1·68	2·43	2·50	2·21
5·0	0	2·28	1·83	1·85	2·45	2·70	2·14
	2	2·12	1·90	1·60	2·51	2·53	2·15
Mean		2·20	1·89	1·73	2·48	2·62	2·15
S.E.M.*		—	—	0·14 (14)	0·18 (14)	—	—

* Standard errors of the means. The numbers in parenthesis are the number of degrees of freedom.

In further tests to determine the distribution of isotope and ions in the lactose, four levels of the lactose layer were assayed separately. The highest concentration of isotope and ions was found near the supernatant (125% of mean concentration) and the concentration of all three progressively fell and was least proximal to the plug (80% of mean concentration in lactose). From the above values it was calculated that, after centrifuging, the extracellular concentration of label and ions in the spermatozoal plug was approximately 5–8% of that in the incubation diluent. Under the experimental conditions in the present studies, this probably does not add a serious error to the estimation of the concentration of intracellular constituents.

III. RESULTS

The effects of various levels of potassium and magnesium ions in the diluent on the metabolism of washed ram spermatozoa with fructose (15 μmoles per flask) or sodium acetate (45 μmoles per flask) as substrate are summarized in Table 2, with

TABLE 3

SUMMARIES OF THE ANALYSES OF VARIANCE OF THE DATA IN TABLE 2

Source of Variation	Experiment 1			Experiment 2			Experiment 3		
	Degrees of Freedom	Variance Ratios		Degrees of Freedom	Variance Ratios		Degrees of Freedom	Variance Ratios	
		Oxygen Uptake	Substrate Oxidized		Oxygen Uptake	Substrate Oxidized		Oxygen Uptake	Substrate Oxidized
Effect of Mg^{2+} (A)									
Linear	1	1·45	1·88	1	14·02**	5·98*	1	9·74**	18·01**
Quadratic	—			1	0·32	0·69	—		
Cubic	—			1	0·32	0·30	—		
Effect of K^{+} (B)									
Linear	1	32·74**	47·12**	1	143·39**	89·16**	1	83·65**	152·74**
Quadratic	1	15·19**	10·98**	—			1	2·42	14·04**
Cubic	—			—			1	4·76*	21·10**
Effect of substrate (C)	1	0·04	0·28	1	5·42*	4·69*	1	1·04	2·34
Interactions									
$A \times B$									
Linear × linear	1	8·43**	4·58*	1	26·12**	5·43*	1	11·43**	6·12*
Other	1	8·48**	0·77	2	1·78	0·98	2	1·04	2·20
$A \times C$	1	0·76	0·20	3	0·04	0·88	1	0·48	0·00
$B \times C$									
Linear × linear	1	1·56	12·69**	1	51·0**	20·37**	1	12·83**	27·50**
Other	1	0·08	0·36	—			2	0·14	2·17
$A \times B \times C$	2	0·08	0·48	3	0·38	0·44	3	0·10	0·00
Ejaculate differences	2	32·10**	24·38**	2	77·23**	12·00**	2	12·29**	18·36**
Ejaculate interactions (error)	22	0·032	0·034	30	0·012	0·030	30	0·066	0·036

* $P < 0·05$. ** $P < 0·01$.

the summaries of the analyses of variance for oxidative metabolism in Table 3. In order to conserve space, the statistical analyses for fructose utilized and lactate accumulated are not tabulated, but the standard errors of the means are given, together with the associated degrees of freedom.

In the first experiment, washed ram spermatozoa ($3–7 \cdot 5 \times 10^8$ cells per flask) were incubated in the presence of a wide range of potassium concentrations, either in the presence or in the absence of 3 mM magnesium. Potassium (5 mM) stimulated total oxygen uptake and substrate oxidation. However, an increase in potassium concentration to 15 mM did not cause a further rise in oxidative metabolism. There

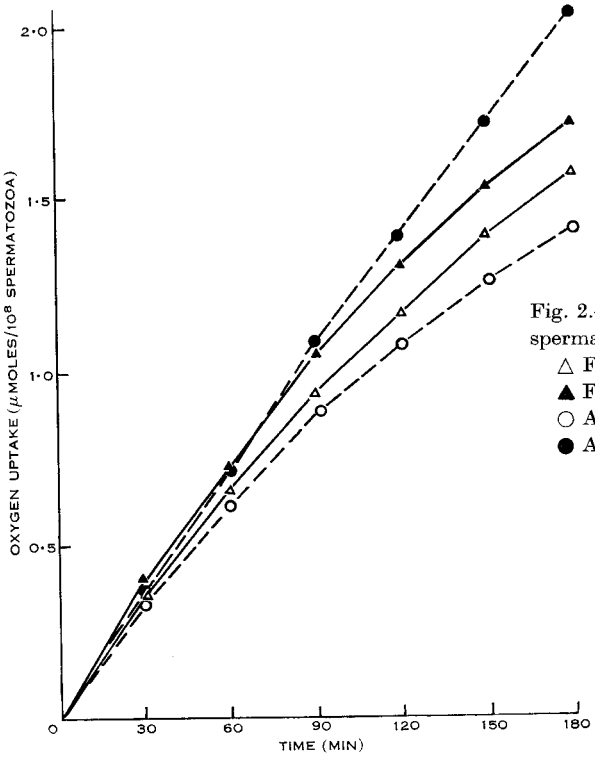

Fig. 2.—Oxygen uptake of washed ram spermatozoa:
△ Fructose diluent.
▲ Fructose diluent containing 5 mM K^+.
○ Acetate diluent.
● Acetate diluent containing 5 mM K^+.

was an interaction between the presence of potassium and the particular substrate available. In the absence of potassium, substrate oxidation by spermatozoa was slightly lower when acetate was substrate than when fructose was available, but, in the presence of potassium, substrate oxidation in diluents containing acetate was higher than that observed in a fructose-containing diluent. Potassium had a greater effect on fructolysis than on fructose oxidation. While fructose oxidation was increased 15–20%, fructose utilization and lactate accumulation were almost doubled. In the absence of potassium, magnesium stimulated metabolism, but in the presence of potassium, magnesium did not cause an effect on substrate oxidation.

In the second experiment, the effect of a range in magnesium concentration, with and without the addition of 5 mM potassium, was tested on three ejaculates of

TABLE 4

METABOLISM OF RAM SPERMATOZOA AND INTRACELLULAR LEVELS OF POTASSIUM, MAGNESIUM, AND SUBSTRATE CARBON FOR SPERMATOZOA INCUBATED FOR 1 HR IN DILUENTS WITH AND WITHOUT THE ADDITION OF POTASSIUM AND MAGNESIUM, WITH FRUCTOSE OR ACETATE AS SUBSTRATE

Values are the means for four ejaculates

Substrate	Ion Added (K^+ 1 mM; Mg^{2+} 2 mM)	Oxygen Uptake (μmoles/10^8 cells)		Final Concn. of K^+ (mg/100 ml)		Final Concn. of Mg^{2+} (mg/100 ml)		Final Concn. of Substrate Carbon (μatoms/ml)	
		Total	From Substrate	Diluent	Cell	Diluent	Cell	Diluent	Cell
Live spermatozoa									
Fructose (5 mM)	Nil	0·94	0·73	1·6	62	0·1	15·2	24	55
	K^+	1·12	0·88	4·5	125	0·1	16·6	24	63
	Mg^{2+}	0·99	0·82	1·1	84	4·1	18·4	25	55
	$K^+ + Mg^{2+}$	1·08	0·85	4·5	130	4·2	19·0	24	56
Acetate (15 mM)	Nil	0·93	0·69	1·1	62	0·1	15·8	26	37
	K^+	1·10	0·89	3·4	123	0·1	16·6	25	28
	Mg^{2+}	1·03	0·81	0·9	69	4·1	18·8	26	27
	$K^+ + Mg^{2+}$	1·07	0·89	3·5	131	4·2	19·2	25	29
Dead spermatozoa									
Fructose (5 mM)	Nil	0·03	0·01	2·6	24	0·3	3·1	27	7
	$K^+ + Mg^{2+}$	0·03	0·01	5·8	31	4·1	10·6	27	8
Acetate (15 mM)	Nil	0·02	0·01	2·6	23	0·3	3·9	29	6
	$K^+ + Mg^{2+}$	0·05	0·00	5·9	25	4·1	8·8	28	7
Unincubated spermatozoa									
Unwashed (diluted)	Nil	—	—	7·1	128	0·6	21·2	—	—
Washed	Nil	—	—	0·8	98	0·1	21·4	—	—

washed ram spermatozoa (4–8 $\times 10^8$ cells per flask). As in the preceding experiment, potassium increased all parameters of metabolism, there was a substrate $\times$ potassium interaction, and potassium had a greater effect when acetate rather than fructose was substrate. In the presence of potassium, magnesium failed to cause any increase in metabolism and when 8 mM magnesium was present there was even a slight fall in fructose utilization. In the absence of potassium, there was a linear increase in all parameters of metabolism measured as the concentration of magnesium increased.

In the third experiment, the effect of a low range in potassium concentration (0–5 mM) with and without the addition of 2 mM magnesium on the metabolism of three ejaculates of washed ram spermatozoa (2·5–4 $\times 10^8$ cells per flask) was studied. Again the addition of potassium stimulated all parameters of metabolism. The stimulation produced by the addition of 1 mM potassium was equal to or greater than that produced by 5 mM potassium. The substrate $\times$ potassium interaction for oxygen uptake and substrate oxidation was similar to that found in the previous experiments. Magnesium again stimulated oxidative metabolism in the absence of potassium, but in the presence of higher levels of potassium there was no effect of magnesium.

The greater stimulation of oxygen uptake by potassium when acetate rather than fructose was substrate was due to differences in the maintenance of oxygen uptake rather than to differences in stimulation during the early part of incubation. The oxygen uptake of spermatozoa incubated over a 3-hr experimental period in the presence or absence of 5 mM potassium is shown in Figure 2. During the first hour, there was little difference between treatments. After this time the rate of oxygen uptake of spermatozoa tended to decline, except in the diluent containing both acetate and potassium, where the rate of oxygen consumption after 3 hr was almost equal to the initial rate.

The effect of potassium (5 mM) and magnesium (1 mM) on the metabolism of four ejaculates of washed ram spermatozoa (4–10 $\times 10^8$ cells per flask) with glucose or acetate as substrate was studied. There was little difference between the metabolism of spermatozoa when incubated without the addition of ions, whether the substrate source was hexose or acetate. As in the previous experiment, the addition of ions had a greater effect when acetate rather than hexose was substrate.

In the next experiment, the intracellular levels of potassium, magnesium, and substrate carbon were measured in washed ram spermatozoa (7–10 $\times 10^8$ cells per flask) incubated 1 hr in the presence and absence of 1 mM potassium and 2 mM magnesium and with either fructose (15 μmoles per flask) or acetate (45 μmoles per flask) as substrate. Formalin-killed spermatozoa from the same ejaculates were also incubated for 1 hr in similar diluents for comparison with the uptake of live spermatozoa. In addition, both oxygen uptake and substrate oxidation of the ejaculates were measured. The results for four ejaculates are given in Table 4, with the analyses of variance in Table 5.

The intracellular level of potassium was greater than the concentration in the diluent. For washed cells incubated in the absence of potassium, the concentration of this ion fell to two-thirds that in washed, unincubated cells. The addition of 1 mM potassium to the diluent, however, doubled the intracellular level and restored it to that found in unwashed, diluted spermatozoa. There was much less change in

the intracellular concentration of magnesium. Washing in a magnesium-free diluent did not change the level of magnesium in the cell and the presence of this ion in the diluent during incubation of washed spermatozoa only increased the levels in the spermatozoa by 20%.

As in the previous experiments, potassium and, to a lesser extent, magnesium, increased oxygen uptake and substrate oxidation. There was also an interaction between the ions such that their effects on metabolism were not additive. At the end of the 1-hr incubation, the intracellular levels of substrate carbon were lower when acetate rather than fructose was the source of substrate. Potassium increased intracellular substrate carbon when fructose was present, but only in the absence of

TABLE 5

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

Source of Variation	Degrees of Freedom	Variance Ratios				
		Total Oxygen Uptake	Oxygen Uptake from Substrate	Intracellular K^+	Intracellular Mg^{2+}	Intracellular Substrate Carbon
Effect of K^+ (A)	1	81·14**	35·19**	274·00**	0·47	0·23
Effect of Mg^{2+} (B)	1	3·18	5·09*	9·27**	5·68*	5·50*
Difference between substrates (C)	1	0·00	0·03	1·31	0·08	220·16**
Interactions						
$A \times B$	1	17·89**	9·08**	1·23	0·06	0·23
$A \times C$	1	1·26	1·87	1·01	0·03	5·42*
$B \times C$	1	1·48	0·74	0·80	0·00	0·12
$A \times B \times C$	1	0·42	0·00	1·72	0·00	6·20*
Replicate differences	3	145·70**	18·10**	9·82**	0·66	3·00
Replicate interactions (error)	21	0·0014	0·0032	97·4	10·7	25·7

*$P < 0·05$. **$P < 0·01$.

magnesium. On the other hand, both potassium and magnesium, alone, decreased intracellular substrate carbon after incubation in the diluents containing acetate. However, the depression caused by a combination of potassium and magnesium was no greater than that occurring in the presence of either ion alone.

Intracellular and extracellular levels of lactate were measured in washed spermatozoal suspensions incubated 1 hr in fructose diluent (15 μmoles per flask) with and without the addition of potassium chloride (1 mM). Mean values from four ejaculates are as follows:

	Lactate Concentration (μmoles/ml)	
	Intracellular	Extracellular
No potassium	2·40	2·14
Potassium present	2·66	3·23

Statistical analysis showed that the addition of potassium significantly increased both intra- and extracellular levels of lactate. The lactate concentration in sperma-

tozoa did not differ significantly from that in the diluent and there was no interaction between potassium addition and lactate distribution.

IV. Discussion

The level of potassium that caused maximum stimulation to metabolism is somewhat lower than levels used in previous studies. The Ringer diluent of Mann (1954) contains 6 mM potassium and in most studies on the effects of this ion on spermatozoa at least 3 mM potassium has been used as the lower limit of concentration (Lardy and Phillips 1943; White 1953*a*, 1953*b*; Wales and White 1958*a*, 1958*b*; Cragle and Salisbury 1959; Wallace and Wales 1964; Wales and Wallace 1964; Wales 1965). The return of the intracellular level of potassium in washed spermatozoa to that of unwashed cells during incubations in diluents containing 1 mM potassium (see Table 4) indicates a very efficient transport of potassium and probably explains the low requirement for this ion.

From the present experiments, it is evident that the addition of magnesium is beneficial to ram spermatozoa, at least when extracellular magnesium has been severely depleted by extensive washing. Since the rate of metabolism in the presence of potassium plus magnesium was no greater than that with potassium alone, magnesium may only be rate-limiting to metabolism in the absence of adequate potassium. The absence of an effect of magnesium in earlier work (Wallace and Wales 1964) may have been due to the composition of diluents used, or to the mild washing procedure employed. As pointed out by Wales (1965), the interaction of magnesium with other variables may explain the varying reports of its effects on the metabolism of spermatozoa.

Methods for the measurement of intracellular levels of substrates suffer from the disadvantage that preparative procedures following incubation are time-consuming and may allow post-incubation changes to occur (see Flipse 1962). The present method overcomes this disadvantage and allows spermatozoa to be collected and their metabolism terminated rapidly after incubation. The method is also useful for the measurement of intracellular ion levels. The low levels of ions and isotope in the lactose rinse solution and the lack of correlation between these levels and intracellular concentrations indicate that exchange of substances between spermatozoa and lactose during centrifugation is minimal and unlikely to introduce any serious errors into the assay of the sperm-plug constituents.

The intracellular concentrations of potassium and magnesium that were found for unwashed diluted spermatozoa are somewhat higher than, but of the same order as, those found by Quinn, White, and Wirrick (unpublished data). These latter workers used a diluent containing four times the concentration of phosphate. Orthophosphate has been shown to depress mitochondrial potassium levels (Stanbury and Mudge 1953; Gamble 1957) and differences in phosphate levels between diluents could explain the discrepancies in the two results. However, the present results confirm the observation of Quinn, White, and Wirrick (unpublished data) that the amount of potassium and magnesium in spermatozoa falls during incubations of ram spermatozoa in the absence of added ions.

Since intracellular levels of substrate carbon were higher when fructose, rather than acetate, was present, a small accumulation of fructose, intermediary metabolite, or end product occurs in spermatozoa during fructolysis. As phosphofructokinase is an important enzyme in regulating the Embden–Meyerhoff pathway (Lowry and Passonneau 1964), hexose phosphate will probably account for some of this accumulated substrate carbon. According to Mann (1964), little fructose is present in spermatozoa, while phosphohexose, which is confined almost exclusively to the cell, is present at three times the concentration of fructose. In addition, the present experiments indicate that there is a rapid equilibration of lactate across the sperm membrane. Thus the contribution of lactate to intracellular substrate carbon will be determined by the total lactate present.

In the glycolytic pathway, there are several metal-activated enzymes, some of which have multiple metal activations (Wyatt 1964). In the present experiments, there was an increase in the intracellular levels of substrate carbon in the presence of potassium, but not when magnesium was also added. This, plus the fact that potassium depressed intracellular substrate carbon with acetate as substrate, points to a substantial effect of potassium on the rate of glycolysis. Possibly increased phosphorylation of fructose in the presence of adequate potassium leads to an accumulation of reaction products. When magnesium is also added, acceleration of later steps removes hexose phosphate and returns the levels of intracellular substrate carbon to normal.

Although potassium and magnesium ions are reported to increase the rate of entry of acetate into the citric acid cycle (Beinert *et al.* 1953; Von Korff 1953), addition of each ion decreased the level of substrate carbon in the cell when acetate was substrate. This indicates an acceleration by these ions in oxidative utilization of substrates by this cycle. Magnesium has a direct effect on the formation of succinic from α-ketoglutaric acid (Krebs and Lowenstein 1960). Although it has not been reported that potassium has a direct effect on the citric acid cycle, its presence may indirectly increase the rate of reaction by increasing the availability of phosphate-acceptor adenosine diphosphate and of inorganic phosphate (see Bilodeau and Elliott 1963). The results of Quinn, White, and Wirrick (1965) suggest the operation of an ion-pump mechanism in spermatozoa, a mechanism which in other tissues is believed to involve the activity of a sodium- and potassium-dependent adenosine triphosphatase (Skou 1957, 1960; Post *et al.* 1960; Dunham and Glynn, 1961).

Flipse (1962) indicated that there was probably an active transport mechanism operating for fructose uptake in spermatozoa. Due to the rapid metabolism of this substrate, this is difficult to prove conclusively and, although the intracellular level of ^{14}C-label in the present study was higher than that in the diluent, this fact need not indicate the accumulation of fructose against a concentration gradient.

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