THE METABOLISM OF RAM, BULL, DOG, AND RABBIT SPERMATOZOA AFTER COOLING TO 5°C

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Summary

The possible occurrence of shunt activity in ejaculated ram spermatozoa was investigated by measuring the amount of labelled carbon dioxide and lactate formed from specifically labelled glucose by the cells. There was no evidence that ram spermatozoa, either shortly after collection or after storage at 5°C, oxidized glucose by the hexose monophosphate pathway.

When casein (4 g/100 ml) was included in a phosphate–saline diluent the oxygen uptake of ram semen after storage at 5°C was greatly improved especially after storage in more dilute suspensions. Washing or centrifuging ram spermatozoa after storage at 5°C slightly decreased their respiration but did not affect fructolysis. Measurement of the ratio of labelled carbon dioxide produced from specifically labelled lactate indicated that less damage occurred if the spermatozoa were allowed to warm to room temperature during washing or centrifuging than if the washing and centrifuging were carried out at 5°C.

With ram and bull semen cooling to 5°C decreased their subsequent metabolism at 37°C and there was a further decrease after storage at 4–5°C. After both cooling and storage, there was less labelled carbon dioxide formed from sodium [2-^{14}C]lactate than from sodium [1-^{14}C]lactate. During cooling to and storage at 4°C ram spermatozoa lost potassium and magnesium and gained calcium. Ram spermatozoa that had been stored at 4°C for 4 days increased their intracellular potassium during incubation at 37°C but the concentration of this ion never returned to that found before cooling. In all cases incubation at 37°C caused a marked accumulation of calcium in the cell. The addition of potassium chloride (5 mM) and magnesium chloride (1 mM) to diluted ram semen during cooling slightly increased cellular metabolism and intracellular potassium and magnesium, and decreased intracellular calcium. In general bull spermatozoa were less affected by the treatments. Cooling to 5°C had minor effects on the respiration of and the potassium concentration in bull spermatozoa and, in this species, most of the depression occurred during the period of storage at 5°C.

Dog and rabbit spermatozoa showed smaller decreases in metabolism due to storage at 5°C. With these species, although there was more labelled carbon dioxide formed from sodium [1-^{14}C]lactate than from sodium [2-^{14}C]lactate there was no change in this relationship following storage at 5°C.

Storage of rabbit semen at 5°C for 20 hr resulted in a marked decline in fertility, but no significant change in the oxidation of lactate.

I. Introduction

The metabolism of spermatozoa after storage at 5°C is less than that of spermatozoa shortly after ejaculation, even when precautions are taken to avoid cold shock (Mann 1964; O'Shea and Wales 1966). In general all measures of metabolism are depressed

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but a differential effect of storage on the subsequent oxidation of carbon atoms 1 and 2 of lactate by bull spermatozoa has been reported (O'Shea and Wales 1966).

In the present paper, further studies on the effects of cooling spermatozoa are reported. In most of these experiments ram semen was used and in particular the oxidation of lactate was examined. Some comparative studies with other mammalian spermatozoa are included.

II. Materials and Methods

(*a*) *General*

Ram semen was collected after electrical stimulation (Blackshaw 1954). An artificial vagina was used for the collection of bull and rabbit semen (Walton 1933; White 1955). Massage was used for the collection of dog semen, the second (or sperm-rich) fraction being used. During collection ejaculates were protected from sudden temperature changes and only apparently normal ejaculates with good initial motility were used. Experiments were started within 1 hr of semen collection.

Semen washed shortly after collection was treated as previously described (O'Shea and Wales 1965). However, the washing procedure for semen that had been stored at 5°C was modified as follows. All operations were carried out at 5°C using pre-cooled apparatus and materials. The diluted semen, in which the spermatozoa had formed a loose plug in the bottom of the tube during storage, was centrifuged for 5 min at 400 *g* and the supernatant replaced with an equal volume of basic diluent. The spermatozoa were dispersed and recentrifuged for 7 min. The second supernatant was removed and the spermatozoa dispersed in diluent.

The basic diluent 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. When other chemicals were included in the diluent, isotonicity was maintained at 308 milliosmoles per litre by decreasing the sodium chloride content.

Spermatozoal counts were estimated by direct counts in a haemocytometer and substrate oxidation was measured by the use of isotopically labelled substrates (O'Shea and Wales 1965). The DL-[2,3-^{14}C]lactate (sodium salt) was obtained from Nuclear Research Chemicals Inc., Florida, U.S.A., and the other labelled compounds were obtained from the Radiochemical Centre, Amersham, England.

The methods of cooling and storing semen, Warburg incubation, measurement of $^{14}CO_2$ by precipitation as $Ba^{14}CO_3$, estimation of fructose and lactate in protein-free extracts, and measurement of amounts of intracellular ions have been described (O'Shea and Wales 1964, 1965; Wales and O'Shea 1966). In the tables the carbon dioxide formation as measured by [2,3-^{14}C]lactate has been divided by two in order to make it comparable with the [1-^{14}C]- and [2-^{14}C]lactate values. In all experiments diluted semen (0·9 ml) and isotopically labelled substrate in 0·9% saline (0·1 ml) were incubated at 37°C for 3 hr in flasks of 5 ml volume containing carbon dioxide-free KOH (20% w/v) in the centre well.

For the final experiment female albino rabbits were inseminated with 0·2 ml of diluted semen following the intravenous administration of 25 i.u. of human chorionic gonadotropin (Wales, Martin, and O'Shea 1965).

(*b*) *Investigation of Hexose Monophosphate Pathway*

This was carried out by comparison of the amount of $^{14}CO_2$ formed from [1-^{14}C]glucose and from [6-^{14}C]glucose (Bloom and Stetten 1953) and by comparison of the ^{14}C activity of the lactate accumulated (Blumenthal, Lewis, and Weinhouse 1954). The radioactivity of the lactate in the protein-free extracts was measured after removal of glucose by two $CuSO_4$–$Ca(OH)_2$ extractions (Barker and Summerson 1941).

In a preliminary experiment with spermatozoal suspensions, to which known amounts of isotopically labelled glucose or lactate were added, precipitation with $Ba(OH)_2$–$ZnSO_4$ and two extractions with $CuSO_4$–$Ca(OH)_2$ were carried out. The recovery of lactate (78% of total) was constant for either washed or unwashed semen. There was a small residual count due to labelled glucose (0·8% of added label) and, in the experiment reported, aliquots of unincubated semen, to which was added labelled glucose, were extracted and used as background for each treatment. Liquid scintillation counting was used to check the level of radioactivity in the materials used and in the $CuSO_4$–$Ca(OH)_2$ extracts. In general, 0·1 ml of an aqueous solution was added directly to 10 ml of toluene–ethanol (2 : 1 v/v) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene obtained from the Packard Instrument Company.

(*c*) *Statistical Analysis*

Most experiments were of factorial design and the results were subjected to standard analyses of variance, which, where presented, are in summary form giving only degrees of freedom and variance ratios for each source of variation with the error mean square in italics at the base of each variance ratio column. Data expressed as percentages were converted to angles before statistical analysis was carried out. In the tables non-significant interactions have been combined and further to conserve space some of the larger analyses of variance are not presented in the tables. In such cases, the standard errors of the means calculated from the analysis of variance are given with the associated degrees of freedom and the statistical significance of the results is quoted in the text.

III. Results

(*a*) *Experiment 1: Metabolism of Specifically Labelled Glucose*

The possible occurrence of the hexose monophosphate pathway in ejaculated ram semen was investigated in fresh semen and in semen stored at 5°C for 44 hr. Aliquots were incubated with and without washing and the metabolism of semen stored at 5°C was compared with that of semen incubated shortly after collection. All diluents used in the experiment contained potassium chloride (5 mM) and magnesium chloride (2 mM). The initial dilution (1 : 9) was made with a diluent containing glucose (4 mM) and casein (4 g/100 ml) and before Warburg incubation a small amount of a diluent containing glucose (80 mM) was added so that aliquots from the same ejaculate contained similar amounts of glucose (about 16 μmoles/flask) and numbers of spermatozoa (average $2{\cdot}75 \times 10^8$ cells/flask). Aliquots were incubated for 1 or 3 hr in the presence of 500 nc/flask of [U-^{14}C]glucose, or [1-^{14}C]glucose, or [6-^{14}C]glucose.

Mean results of $^{14}CO_2$ evolved and labelled lactate formed are given in Table 1. There was no significant difference in the amounts of labelled carbon dioxide or lactate formed from [1-^{14}C]glucose and [6-^{14}C]glucose during any of the incubations. However, the use of uniformly labelled glucose gave more $^{14}CO_2$ than the use of specifically labelled glucose ($P<0\cdot01$). The 3 hr incubation gave more $^{14}CO_2$ and labelled lactate than the 1 hr incubation ($P<0\cdot05$), and the increase in $^{14}CO_2$ production due to washing was greater with the 3 hr incubation than with the 1 hr incubation ($P<0\cdot01$). Stored spermatozoa, when unwashed, formed less labelled lactate than did the unwashed control suspensions ($P<0\cdot01$).

TABLE 1

EFFECT OF STORAGE AT 5°C FOR 44 HR, WASHING, AND TIME INCUBATED ON THE OXIDATION OF GLUCOSE BY RAM SPERMATOZOA

Values are expressed per 10^8 spermatozoa over the experimental period and are the means for three ejaculates

Time of Incubation (hr)	Labelled Glucose Used	$^{14}CO_2$ Produced (nc)		[^{14}C]Lactate Accumulated (counts/min)*	
		Control	Stored	Control	Stored
		Cells not washed			
1	U-^{14}C	1·94	0·90	113	102
	1-^{14}C	1·66	0·59	124	108
	6-^{14}C	1·60	0·55	111	95
3	U-^{14}C	5·81	3·69	230	194
	1-^{14}C	4·92	3·27	236	187
	6-^{14}C	4·97	3·07	238	201
		Cells washed			
1	U-^{14}C	4·46	3·29	114	117
	1-^{14}C	3·36	2·44	122	122
	6-^{14}C	3·28	2·36	107	120
3	U-^{14}C	8·60	7·87	218	229
	1-^{14}C	7·13	7·03	222	226
	6-^{14}C	7·85	7·19	222	240
Standard error of the mean		0·45†		11·7†	

* Per 0·1 ml $CuSO_4$–$Ca(OH)_2$ extract. † 45 degrees of freedom.

(*b*) *Experiment 2: Effect of Dilution, Casein, and Storage on Ram Semen*

The effect of dilution, casein, and storage at 5°C on the oxygen utilized and the carbon dioxide produced from carbons 1 and 2 of lactate by ram spermatozoa was investigated. Ram semen was diluted in 9, 19, and 39 volumes of the basic diluent containing fructose (10 mM), with and without the addition of casein (4 g/100 ml). Aliquots were incubated immediately or after storage at 5°C for 20 hr. Replicate flasks contained [U-^{14}C]fructose (50 nc), DL-[1-^{14}C]lactate (80 nc), or

DL-[2-^{14}C]lactate (60 nc). The results for three ejaculates (mean $2{\cdot}8 \times 10^8$ cells/flask in the most concentrated treatment) are summarized in Table 2. Neither casein nor dilution had marked effects on the oxygen uptake of fresh aliquots during the 3 hr incubation. However, the oxygen uptake of samples stored without the addition of casein to the diluent was greatly depressed, especially in the more dilute suspensions. The presence of casein during storage at 5°C greatly increased subsequent oxygen consumption by the cells ($P<0{\cdot}01$) and it almost returned to that of the control (unstored) suspensions. The effect of casein was most evident in the more dilute suspensions ($P<0{\cdot}05$). The ratio of the CO_2 formed from carbon 2 as compared with that from carbon 1 of lactate was decreased by storage in the absence of casein, but was not altered by storage when casein was added ($P<0{\cdot}01$).

TABLE 2

EFFECT OF DILUTION RATE, ADDITION OF CASEIN, AND STORAGE AT 5°C FOR 20 HR ON THE METABOLISM OF RAM SPERMATOZOA

Values are the means for three ejaculates over the experimental period (3 hr). The $^{14}CO_2$ ratio is the $^{14}CO_2$ formed from [2-^{14}C]lactate expressed as a percentage of the $^{14}CO_2$ formed from [1-^{14}C]lactate

Dilution	Oxygen Uptake (μmole/10^8 cells)		$^{14}CO_2$ Ratios	
	Control	Stored	Control	Stored
	Casein absent			
10	2·49	0·74	81·6	56·7
20	2·60	0·58	82·1	56·9
40	2·62	0·47	80·2	49·3
	Casein present			
10	2·64	1·75	81·8	72·9
20	2·75	2·05	86·2	79·8
40	2·57	2·19	81·5	79·1
Standard error of the mean	0·253*		3·23*	

* 22 degrees of freedom.

(c) *Experiment 3: Effect of Washing on Cooled Ram Spermatozoa*

The effect of washing spermatozoa after storage at 5°C in diluents containing casein (4 g/100 ml) was examined in detail. Ram semen was diluted with 9 volumes of a diluent containing fructose (4 mM) and stored at 5°C for 20 hr. Aliquots were then incubated at 37°C after the following treatments: (1) stored but unwashed (control); (2) washed at 5°C as described in Section II(*a*); (3) centrifuged twice at 5°C with dispersal in the supernatant after centrifuging; (4) washed at room temperature: the diluted stored semen was washed as described for cooled semen in Section II(*a*) except that the temperature of the sample and washing diluent was allowed to rise to room temperature during the first centrifuging and subsequent

manipulations were carried out at room temperature; (5) centrifuged twice at room temperature with dispersal in the supernatant. Fructose and lactate determinations were carried out on the original semen as soon as it reached 5°C, and all treatments were adjusted to have approximately equal amounts of fructose (mean 6 μmoles/flask), lactate (mean 2·5 μmoles/flask), and numbers of spermatozoa (mean $3{\cdot}6\times10^8$

TABLE 3

EFFECT OF WASHING ON THE METABOLISM OF RAM SPERMATOZOA AFTER STORAGE AT 5°C FOR 20 HR

Values are the means for four ejaculates over the experimental period (3 hr) and the oxygen, fructose, and lactate data are expressed as μmole/10^8 spermatozoa. The $^{14}CO_2$ ratio is the $^{14}CO_2$ formed from [2-^{14}C]lactate expressed as a percentage of the $^{14}CO_2$ formed from [1-^{14}C]lactate

Treatment	Oxygen Uptake	$^{14}CO_2$ Ratio	Fructose Utilized	Lactate Accumulated
Control	1·20	77·7	0·68	1·05
Centrifuged 5°C	1·17	71·9	0·68	1·04
Washed 5°C	0·96	62·3	0·68	0·83
Centrifuged room temperature	1·13	74·4	0·70	1·01
Washed room temperature	1·07	71·3	0·62	1·00

Summary of the Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios			
		Oxygen Uptake	$^{14}CO_2$ Ratio	Fructose Utilized	Lactate Accumulated
Treatment (*A*)					
Control *v.* others (1)	1	2·78	5·49*	0·09	0·90
Centrifuged *v.* washed (2)	1	4·24	4·16	0·94	2·16
5°C *v.* room temperature (3)	1	0·11	4·05	0·20	1·02
(2) × (3)	1	1·50	1·26	0·79	1·85
Ejaculate differences (*B*)	3	86·94**	13·47**	53·13**	51·23**
A × *B*	12	*0·0475*	*14·162*†	*0·0175*	*0·0647*
Within group	40	0·0025	—	0·0037	0·0082

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

† Degrees of freedom reduced by one to allow for missing value.

cells/flask) at the commencement of incubation. The washed spermatozoa were suspended in diluents containing potassium chloride (1 mM) and magnesium chloride (2 mM). The results for four ejaculates (Table 3) show that centrifuging and washing lowered the ratio of $^{14}CO_2$ from carbon atom 2 to $^{14}CO_2$ from carbon atom 1 of lactate. There were no significant effects of the treatments on oxygen uptake, fructose utilization, or lactate accumulation.

(*d*) *Experiments 4 and 5: Effect of Cooling on Bull and Ram Semen*

In experiment 4 a comparison of the effects of cooling to and storage at 4°C on bull semen was made by measuring changes in metabolism and intracellular ions.

TABLE 4

EFFECT OF COOLING TO 4°C AND OF STORAGE AT 4°C FOR 4 DAYS ON THE METABOLISM OF BULL SPERMATOZOA

Values are expressed as μmole/10^8 spermatozoa over the experimental period (3 hr) and are the means for three ejaculates

Treatment	Carbon Dioxide from Position Labelled			Oxygen Uptake	Fructose Utilized	Lactate Accumulated
	[1-^{14}C]Lactate	[2-^{14}C]Lactate	[U-^{14}C]Fructose			
Control	0·18	0·16	0·91	1·60	0·92	1·75
Cooled to 4°C	0·19	0·14	0·71	1·40	0·90	1·54
Stored at 4°C	0·12	0·04	0·10	0·39	0·50	0·80
Standard error of the mean	0·007*		0·069*	0·118*	0·117*	0·19*

* 4 degrees of freedom.

Bull semen was diluted with four volumes of a diluent containing fructose (4 mM) and casein (4 g/100 ml) and the metabolism compared with that of aliquots cooled to

TABLE 5

EFFECT OF COOLING TO 4°C, STORAGE AT 4°C FOR 4 DAYS, AND INCUBATION AT 37°C FOR 3 HR ON THE AMOUNT OF K^+ AND Mg^{2+} IN BULL SPERMATOZOA

Values are expressed as mg/100 ml and are the means for three ejaculates

Treatment	Time at 37°C (hr)	K^+ in Supernatant	K^+ in Cells	Mg^{2+} in Supernatant	Mg^{2+} in Cells
Control	0	22·2	148·5	1·13	15·0
	3	21·6	115·9	1·16	15·4
Cooled to 4°C	0	22·2	128·4	1·13	14·9
	3	20·8	120·9	1·05	11·7
Stored at 4°C	0	21·8	53·7	1·05	13·3
	3	21·4	58·4	1·13	6·4
Standard error of the mean		—	12·3*	—	0·80*

* 10 degrees of freedom.

4°C and also with aliquots stored at 4°C for 4 days. In order to ensure that all samples contained similar amounts of fructose during incubation, one volume of a diluent

TABLE 6

EFFECT OF THE ADDITION OF K^+ AND Mg^{2+}, COOLING TO 4°C, AND STORAGE AT 4°C FOR 4 DAYS ON THE METABOLISM OF RAM SEMEN

Values are the means for four ejaculates expressed as μmole/10^8 spermatozoa over the experimental period (3 hr)

Treatment	$K^+ + Mg^{2+}$	Carbon Dioxide from Position Labelled				Oxygen Uptake	Fructose Utilized	Lactate Accumulated
		[1-^{14}C]-Lactate	[2-^{14}C]-Lactate	[2,3-^{14}C]-Lactate	[U-^{14}C]-Fructose			
Control	−	0·23	0·20	0·19	1·16	2·19	1·52	2·47
	+	0·22	0·19	0·19	1·06	2·19	1·50	2·64
Cooled to 4°C	−	0·20	0·13	0·13	0·33	1·05	0·71	1·38
	+	0·22	0·16	0·15	0·41	1·27	0·89	1·56
Stored at 4°C	−	0·14	0·07	0·07	0·08	0·54	0·45	0·46
	+	0·15	0·09	0·09	0·12	0·62	0·39	0·70
Standard error of the mean			0·008*		0·05†	0·102†	0·075†	0·185†

* 51 degrees of freedom. † 15 degrees of freedom.

containing fructose (30 mM) was added to nine volumes of each sample of diluted semen immediately prior to incubation. Replicate Warburg flasks were run containing [U-^{14}C]fructose (50 nc), DL-[1-^{14}C]lactate (60 nc), or DL-[2-^{14}C]lactate (50 nc). The results (Table 4) for three ejaculates (mean $2{\cdot}1 \times 10^8$ cells/flask) show that cooling to 4°C caused a decrease in the amount of fructose oxidized ($P < 0{\cdot}01$) and storage at 4°C decreased respiration ($P < 0{\cdot}01$) and fructolysis ($P < 0{\cdot}05$). After both cooling to 4°C and storage at 4°C there was a greater formation of CO_2 from carbon atom 1 than from carbon atom 2 of lactate ($P < 0{\cdot}01$). Storage at 4°C (Table 5) caused a decrease in intracellular potassium ($P < 0{\cdot}01$). After storage at 4°C incubation of semen caused a decrease in intracellular magnesium ($P < 0{\cdot}01$). A similar but non-significant change in intracellular magnesium concentration occurred in cooled samples.

TABLE 7

EFFECT OF THE ADDITION OF K^+ AND Mg^{2+}, COOLING TO 4°C, STORAGE AT 4°C FOR 4 DAYS, AND INCUBATION AT 37°C FOR 3 HR ON INTRACELLULAR K^+, Mg^{2+}, AND Ca^{2+} OF RAM SPERMATOZOA

Values are the means for four ejaculates, expressed as mg/100 ml

Treatment	Time at 37°C	Ions Added	K^+ in Supernatant	K^+ in Cell	Mg^{2+} in Supernatant	Mg^{2+} in Cell	Ca^{2+} in Supernatant	Ca^{2+} in Cell
Control	0	—	6·7	107·5	0·6	16·7	1·45	25·8
		+	20·2	120·8	2·2	19·0	1·55	26·7
	3	—	7·2	87·8	0·6	12·5	1·39	86·3
		+	20·6	87·4	2·2	13·5	1·33	83·7
Cooled to 4°C	0	—	7·9	37·7	0·6	10·5	1·35	55·3
		+	20·4	49·6	2·2	14·7	1·31	42·8
	3	—	7·6	36·1	0·6	9·2	1·30	121·6
		+	21·7	54·0	2·2	15·2	1·37	99·0
Stored at 4°C	0	—	7·0	19·4	0·7	6·0	1·47	40·5
		+	20·6	22·6	2·2	7·4	1·42	38·2
	3	—	7·7	26·8	0·7	6·6	1·26	115·2
		+	21·4	31·3	2·2	9·0	1·22	107·7
Standard error of the mean			—	5·77*	—	0·95*	—	6·57*

* 33 degrees of freedom.

A similar experiment was performed using ram spermatozoa. Aliquots of ram semen were diluted with nine volumes of diluents containing fructose (4 mM) and casein (4 g/100 ml) either with or without the addition of both potassium chloride (5 mM) and magnesium chloride (1 mM). The metabolism of aliquots incubated immediately after collection was compared with that of aliquots incubated at 37°C immediately after cooling to 4°C and after storage at 4°C for 4 days. As in experiment 4, a small amount of a diluent containing fructose (30 mM) was added to each suspension immediately prior to incubation. The results for four replicates (mean $2{\cdot}3 \times 10^8$ cells/flask) are presented in Tables 6 and 7. There was no difference in the oxidation of sodium [2-^{14}C]lactate and sodium [2,3-^{14}C]lactate. Although there was no

TABLE 8

EFFECT OF STORAGE AT 5°C FOR 20 HR ON THE OXIDATIVE METABOLISM OF DOG SEMEN AND ON THE OXIDATIVE METABOLISM AND FERTILITY OF RABBIT SEMEN

Metabolic values are expressed as μmole/10^8 spermatozoa over the experimental period (3 hr). Six does were inseminated per group per ejaculate. Values are the means for four ejaculates (dog) and for three ejaculates (rabbit)

Treatment	No. of Litters	Litter Size	Carbon Dioxide from Position Labelled				Oxygen Uptake
			[1-^{14}C]Lactate	[2-^{14}C]Lactate	[2,3-^{14}C]Lactate	[U-^{14}C]Fructose	
			Dog				
Control			0·201	0·146	0·135	0·72	2·25
Stored at 5°C			0·196	0·136	0·136	0·41	1·50
Standard error of mean				0·004		0·25	0·15
Degrees of freedom				15		3	3
			Rabbit				
Control	5·7	5·9	0·112	0·107	0·080	0·370	0·93
Stored at 5°C	2·7	5·1	0·123	0·093	0·077	0·288	0·87
Standard error of mean	0·53	0·45		0·01		0·01	0·02
Degrees of freedom	2	2		10		2	18

significant difference in the amount of carbon dioxide formed from the various carbons of lactate by unchilled suspensions, cooling gave a decrease in the formation of carbon dioxide from carbon atom 2 and from carbon atoms 2 and 3, but not in carbon dioxide from carbon atom 1 ($P<0\cdot01$). Storage at 4°C caused a further reduction in the formation of carbon dioxide from carbon atom 2 and from carbon atoms 2 and 3 of lactate ($P<0\cdot05$). Addition of potassium plus magnesium ions stimulated the oxidation of lactate ($P<0\cdot01$) and of fructose plus lactate ($P<0\cdot05$) by semen that had been cooled or stored. However, the amount of lactate oxidized, fructose oxidized, fructose plus lactate oxidized, oxygen uptake, and fructolysis were decreased by cooling and further decreased by storage ($P<0\cdot01$). The concentration of potassium in the spermatozoa was markedly depressed by cooling to and by storage at 4°C ($P<0\cdot01$). This treatment also caused a fall in the intracellular level of magnesium ($P<0\cdot01$) and cooling caused a rise in calcium concentration ($P<0\cdot01$). The addition of potassium and magnesium ions to the diluent increased intracellular potassium ($P<0\cdot05$) and magnesium ($P<0\cdot01$) and decreased calcium levels ($P<0\cdot05$). However, in general, the effects of the addition of ions to the diluent were small when compared with the effects of storage and their addition did little to offset the movement of intracellular ions caused by storage. Although incubation for 3 hr depressed the potassium and magnesium concentration in fresh samples ($P<0\cdot01$), it did not affect the low levels of these ions found in cooled or stored cells. In all cases, incubation at 37°C greatly increased intracellular calcium concentration ($P<0\cdot01$).

(e) *Experiments 6 and 7: Effect of Storage on Dog and Rabbit Semen*

The effect of storage at 5°C on the metabolism of dog semen was investigated in experiment 6. Dog semen was diluted 1 : 1 in a diluent consisting of 40 mM mono- and disodium phosphate buffer (pH 7·0), fructose (4 mM), sodium lactate (2 mM), sodium chloride (96 mM), penicillin (60 mg/100 ml), streptomycin (100 mg/100 ml) and casein (8 g/100 ml). The metabolism of aliquots of four ejaculates (mean $1\cdot6\times10^8$ cells/flask) after storage for 20 hr at 5°C was less than that of control samples incubated shortly after collection (Table 8). The amount of CO_2 produced from carbon atom 1 of lactate was significantly greater than that from the other carbon atoms in both control and stored semen ($P<0\cdot01$).

A similar experiment was carried out with rabbit semen (expt. 7). In order to measure accurately lactate oxidation, it was found necessary to increase the specific activity of the lactate by halving the lactate concentration in the diluent used in experiment 6.

In addition to measuring the metabolism of the spermatozoa aliquots were diluted further with nine volumes of diluent and six does inseminated with 0·2 ml of the diluted semen from each treatment (mean $6\cdot6\times10^6$ spermatozoa/doe). The results for three ejaculates (mean $3\cdot0\times10^8$ cells/flask) are given in Table 8. Although storage decreased the number of does littering and the oxygen uptake of the spermatozoa, there was no significant effect of storage on the relative production of carbon dioxide from the carbon atoms of lactate. Again carbon atom 1 of lactate formed more carbon dioxide than did either of the other carbon atoms ($P<0\cdot05$).

IV. Discussion

There is evidence that mammalian spermatozoa utilize glucose by the hexose monophosphate pathway in certain circumstances. Thus, after incubation in the rabbit uterus, rabbit spermatozoa appear to oxidize glucose by the hexose monophosphate pathway (Mounib and Chang 1964; Murdoch and White 1967). It has been reported that testicular spermatozoa also metabolize glucose by this pathway (Wu *et al.* 1959). Glucose-6-phosphate dehydrogenase has been shown to occur in ram and bull spermatozoa (Blackshaw 1963) but, despite the brief report of Flipse (1956), the present work indicates that the pentose pathway is not utilized by ejaculated ram spermatozoa whether fresh or stored. Scott, White, and Annison (1962) also found no evidence of shunt activity in ejaculated mammalian spermatozoa. The greater formation of $^{14}CO_2$ from [U-^{14}C]glucose than from either [1-^{14}C]glucose or [6-^{14}C]glucose is due to the 1 and 6 positions of glucose forming carbon atom 3 of lactate which forms less $^{14}CO_2$ than uniformly labelled lactate. The increase in production of $^{14}CO_2$ caused by washing in this experiment is due to the removal of endogenous lactate and fructose which dilute the labelled substrate.

As previously shown by O'Shea and Wales (1966) casein was very effective in offsetting the detrimental effect of storage at 5°C on the respiration of ram spermatozoa. In addition to maintaining total respiration it was effective in increasing the oxidation of carbon atom 2 of lactate relative to carbon 1. Although the precise mechanism of the action of casein is unknown, it has been shown to be protective against the effects of rapid cooling (Choong and Wales 1962). Proteins and other large molecules have been found to protect spermatozoa against the effects of dilution (Blackshaw 1953; Wales and White 1961, 1963) and it has been suggested that their beneficial effect is due to maintenance of the integrity of the cell membrane. In the present experiment casein was more effective in maintaining the metabolism of more dilute suspensions during storage. A previous report (O'Shea and Wales 1964) that the motility of ram spermatozoa after storage at 5°C in a casein diluent was better in more concentrated suspensions indicates a possible differential effect on motility and metabolism. However, as there were differences in dilution rates and diluents used in the two studies, a direct comparison cannot be made.

The metabolism of both ram and bull spermatozoa is depressed by cooling or storage at 5°C. The effect is less in the bull and the magnitude of the depression seems to parallel the extent to which potassium is lost from the cell during treatment. A fall in the ratio of $^{14}CO_2$ formed from [2-^{14}C]lactate to $^{14}CO_2$ from [1-^{14}C]lactate also occurs during cooling and storage. Although this decrease was not significant in some experiments with ram semen (O'Shea and Wales 1966) the fall in the ratio appears to be related to cell damage as indicated by depressed respiration and intracellular potassium and is decreased by the presence of casein during cooling. There was no significant fall in this ratio on cooling dog and rabbit semen and, in the rabbit, the ratio is not related to changes in the fertility of semen samples.

As previously reported (O'Shea and Wales 1966) the addition of small amounts of potassium and magnesium during cooling and storage was beneficial to the metabolism of ram spermatozoa. This may be related to the parallel rise in intracellular potassium and magnesium and depression of intracellular calcium that

occurs on the inclusion of the ions in the diluent before storage. However, the intracellular levels of potassium and magnesium are still very depressed in the presence of the ions and presumably the treatment has damaged ion transport mechanisms. That the amount of intracellular potassium is dependent on an active process (see Quinn, White, and Wirrick 1965) is supported by the finding that intracellular potassium falls during storage and subsequently increases slightly during incubation at 37°C when energy would again be available.

The accumulation of calcium in the cell during cooling and storage is insufficient to account for the decrease in potassium and magnesium and, to preserve ionic equilibrium, presumably sodium also enters, as has been observed after cold shock and deep freezing (Quinn and White 1966). During incubation at 37°C, however, calcium must accumulate at the expense of sodium and becomes a major intracellular cation with concentrations between 40 and 60 m-equiv/l.

The concentration of potassium in bull spermatozoa is less affected by storage at 5°C than is the level of this ion in ram spermatozoa. When compared with controls, cooling to 5°C caused a loss of 65% of the potassium from ram spermatozoa and this increased to 82% following storage at 5°C for 4 days. The comparable values for bull spermatozoa are 13% and 61% respectively. On the other hand there was no evidence of an accumulation of this ion during subsequent incubation, although in another experiment (O'Shea and Wales, unpublished data) a rise in intracellular potassium occurred during incubation of samples of bull semen stored for 44 hr at 5°C rather than 72 hr.

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