METABOLISM OF SEMEN AFTER FREEZING

II.* THE AEROBIC METABOLISM OF RAM SPERMATOZOA BEFORE AND AFTER STORAGE AT −79°C

By T. O'Shea†

[*Manuscript received July* 11, 1968]

Summary

The metabolism of ram spermatozoa, incubated at 37°C shortly after ejaculation and after storage overnight at −79°C, was examined using various combinations of fructose, sodium lactate, sodium acetate, and sodium salts of citric acid cycle intermediates. Although freezing depressed all indices of cell metabolism, there were few qualitative differences between the metabolism by fresh or thawed semen of the various substrates. The increased oxygen uptake of thawed spermatozoa on addition of succinate was unrelated to motility and to other parameters of metabolism.

Addition of 25 mM sodium lactate to the incubation medium gave a lower rate of glycolysis and fructose oxidation but had no effect on oxygen uptake at the pH used (7·2). In the presence of acetate or oxaloacetate fresh and stored cells oxidized less fructose and lactate. However, fructose utilization and lactate accumulation by recently ejaculated spermatozoa were higher on addition of oxaloacetate.

With thawed cells significant correlations were found between the fraction of spermatozoa unstained, the oxygen uptake, the amount of labelled carbon dioxide produced from sodium [2-^{14}C]lactate expressed as a fraction of the labelled carbon dioxide produced from sodium [1-^{14}C]lactate, and the lactate accumulated. When the same parameters measured after thawing were expressed as a fraction of the values obtained before freezing, positive correlations were also obtained.

I. Introduction

After frozen storage the limited washing which can be done without undue damage means that spermatozoa suitable for metabolic studies cannot be obtained completely free of metabolic substrates (O'Shea 1969). Under these conditions the main residues present are fructose and lactate. This being so the metabolism by fresh and stored ram spermatozoa of fructose plus lactate, and of acetate and several constituents of the tricarboxylic acid cycle in the presence of fructose plus lactate were studied.

As the standard procedure 6 mM fructose and 0·1 mM sodium L-lactate were added to the incubation media. This seemed to provide sufficient fructose for the metabolism of recently collected semen and small enough amounts of each substance to make practicable accurate measurements of the metabolism of thawed cells.

* Part I, *Aust. J. biol. Sci.*, 1969, **22**, 721–32.

† Department of Veterinary Physiology, University of Sydney, N.S.W. 2006.

II. Materials and Methods

(a) *Preparation of Spermatozoa*

The methods used for collection, freezing, thawing, washing, and incubating the ram spermatozoa have been described (O'Shea 1969). The aliquots of the semen to be frozen were mixed at 25°C with four volumes of a diluent containing 9 g/100 ml skim-milk powder and 17 mM fructose and placed in a refrigerated cabinet and cooled to 5°C over 2 hr. About 2 hr after the diluted semen had reached 5°C an equal volume of a diluent containing 9 g/100 ml skim-milk powder, 140 mM fructose, and 15 ml/100 ml glycerol was added. The semen was kept at 5°C for a further 3 hr before it was frozen to −79°C. This technique was adopted so that the cooling and freezing procedures did not interfere with the incubation at 37°C of control aliquots of the semen. The washing diluent consisted of 20 mM mono- and disodium phosphate buffer (pH 7·2), 1 mM potassium chloride, 2 mM magnesium chloride, and 123 mM sodium chloride. All incubation was carried out using washed spermatozoa which were suspended in the washing diluent containing the appropriate substrates.

(b) *Analytical Methods*

In most experiments the radioactivity of carbon dioxide trapped in the centre well was assayed by precipitation as $Ba^{14}CO_3$, counting with an end-window Geiger–Müller tube, and correcting for self absorption by the method of Hendler (1959). For the final experiments the contents of the centre well were transferred, using 0·8 ml water, and counted by liquid scintillation techniques in 10 ml of a scintillator consisting of toluene–Triton X-100 (2:1) (Patterson and Greene 1965) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene obtained from the Packard Instrument Company.

The techniques used for the assay of lactate and fructose have been described by O'Shea and Wales (1965). Isotopically labelled lactate accumulated from [U-^{14}C]fructose was estimated by paper chromatography of the reaction mixture in a descending system using n-butanol–acetic acid–water (4:1:5). After chromatography 1-cm strips of the lactate peaks were extracted with 0·4 ml of water and counted in 5 ml of toluene–Triton scintillator. The value for accumulated labelled lactate differed from that for lactate accumulated as measured enzymatically because some of the cold lactate present at the start of incubation was oxidized.

The percentage of spermatozoa unstained before incubation was estimated using Congo red–nigrosin dissolved in phosphate buffer.

(c) *Statistical Methods*

When the metabolism of fresh and thawed spermatozoa was compared, the raw data were converted to logarithms before analyses of variance were carried out. To estimate the effects of the addition of succinate, fumarate, or oxaloacetate, Duncan's multiple range test (Duncan 1955) was used to rank the treatments. The data from the common control treatment used in all experiments were used to obtain correlation coefficients (Snedecor 1956) between characteristics of spermatozoa.

III. Results

(a) *Experiment 1: Metabolism of Fructose and Lactate*

The metabolism of washed ram spermatozoa in the presence of a constant level of fructose (6 mM) and increasing amounts of sodium lactate (1, 5, and 25 mM) was examined before and after storage at −79°C. The mean results and summary of analyses of variance for four ejaculates (2–3 $\times 10^8$ cells per flask) are given in Table 1.

Freezing decreased cell metabolism. The increase in lactate oxidation when 1 mM lactate was replaced by 5 mM lactate was greater than the increase when 5 mM was replaced by 25 mM. The rate of this increase in the amount of lactate

TABLE 1

EFFECT OF FREEZING TO −79°C ON THE METABOLISM OF RAM SPERMATOZOA IN THE PRESENCE OF FRUCTOSE (6 mM) AND VARIOUS LEVELS OF ADDED SODIUM LACTATE

Values are expressed as μmoles per 10^8 spermatozoa over the experimental period (3 hr) and are the means for four ejaculates. 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	Lactate Concn. (mM)	Carbon Dioxide from Labelled Position of:			$^{14}CO_2$ Ratio	Oxygen Uptake	Fructose Utilized	Lactate Accumulated	
		[1-^{14}C]Lactate	[2-^{14}C]Lactate	[U-^{14}C]Fructose				Total	From Fructose
Control	1	0·187	0·168	1·860	90	2·95	1·99	2·04	2·26
	5	0·511	0·452	0·862	89	2·94	1·92	1·79	2·22
	25	0·819	0·740	0·243	90	2·87	1·58	1·54	1·69
Frozen	1	0·093	0·042	0·015	·47	0·30			0·10
	5	0·124	0·052	0·005	42	0·32			0·09
	25	0·138	0·045	0·002	33	0·34			0·05

Summary of Analyses of Variance (on Transformed Values)

Source of Variation	D.F.	Variance Ratio: CO_2 from Lactate	D.F.	Variance Ratios: CO_2 from Fructose	$^{14}CO_2$ Ratio	Oxygen Uptake	Lactate Accumulated from Fructose
Freezing (*A*)	1	1,380·51**	1	765·02**	118·46**	11,604·38**	296·36**
Lactate level (*B*):							
Linear (B_1)	1	230·39**	1	87·25**	1·10	3·35	3·98
Quadratic (B_2)	1	14·79**	1	0·61	0·02	0·00	0·87
Lactate label (*C*)	1	130·51**	—				
Interactions:							
$A \times B_1$	1	119·09**	1	0·05	1·54	7·28*	0·67
$A \times B_2$	1	1·76	1	0·24	0·02	0·21	0·15
$A \times C$	1	79·47**	—				
$B \times C$	2	1·17	—				
$A \times B \times C$	2	1·17	—				
Ejaculate differences	3	19·60**	3	1·55	3·54*	65·75**	3·62*
Ejaculate interactions	33	*0·0048*†	15	*0·035*†	*49·75*†	*0·0005*†	*0·0454*†

*$P < 0·05$. **$P < 0·01$. † Error mean squares.

oxidized with added lactate was lower with thawed than with control spermatozoa. A greater fall after storage in the amount of $^{14}CO_2$ formed from [2-^{14}C]lactate than from [1-^{14}C]lactate caused the decrease in the $^{14}CO_2$ ratio with storage. Increased lactate had no effect on the oxygen uptake of freshly collected spermatozoa but caused a slight increase with stored cells of the same ejaculates.

Utilization of fructose by recently ejaculated spermatozoa was unaltered when lactate was increased from 1 to 5 mM but was depressed by 25 mM lactate ($P<0\cdot01$). The same cells showed a linear decrease in total lactate accumulation ($P<0\cdot05$). Lactate accumulation from fructose also fell with 25 mM lactate but the effect was not significant.

(*b*) *Experiment 2: Effect of Acetate Addition on the Metabolism of Substrates*

Ram spermatozoa, either recently ejaculated or after storage overnight at −79°C, were incubated in the presence of constant levels of fructose (6 mM) and sodium lactate (0·1 mM) and increasing amounts of sodium acetate (0, 1, and 25 mM). The mean results for four ejaculates (1–3 $\times10^8$ cells per flask for controls and 1–5 $\times10^8$ cells per flask for stored cells) are given in Table 2.

TABLE 2

EFFECT OF STORAGE AT −79°C ON THE METABOLISM OF RAM SPERMATOZOA INCUBATED IN THE PRESENCE OF FRUCTOSE (6 mM), SODIUM LACTATE (0·1 mM), AND SODIUM ACETATE (0, 1, OR 25 mM)

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

Treatment	Acetate Concn. (mM)	Carbon Dioxide Produced from Labelled Position of:					Oxygen Uptake	Fructose Utilized	Lactate Accumulated	
		[1-^{14}C]-Lactate	[2-^{14}C]-Lactate	[U-^{14}C]-Fructose	[1-^{14}C]-Acetate	[2-^{14}C]-Acetate			Total	From Fructose
Control	0	0·052	0·044	1·872	—	—	2·47	2·13	1·97	1·95
	1	0·043	0·032	1·392	0·345	0·335	2·53	2·20	2·37	2·20
	25	0·046	0·006	0·708	0·614	0·636	2·06	1·96	1·68	1·65
Frozen	0	0·037	0·021	0·048	—	—	0·25		0·05	0·04
	1	0·034	0·013	0·036	0·026	0·025	0·27		0·05	0·04
	25	0·031	0·002	0·018	0·067	0·061	0·28		0·04	0·03

Freezing decreased all parameters of cell metabolism ($P<0\cdot001$). Addition of acetate gave a linear decrease ($P<0\cdot001$) in fructose oxidation and a concomitant increase in acetate oxidized when increased from 1 to 25 mM ($P<0\cdot001$). Again, storage resulted in a larger fall in CO_2 production from the carbon-2 position than from the carbon-1 position of lactate ($P<0\cdot001$). The fall in CO_2 production from lactate with addition of acetate was due to a fall in CO_2 from carbon-2 as there was no decrease in oxidation of carbon-1 ($P<0\cdot001$). A larger decrease in production of $^{14}CO_2$ from [2-^{14}C]lactate occurred when acetate was increased from 1 to 25 mM than when 1 mM acetate was added ($P<0\cdot001$). Addition of acetate had no significant effect on oxygen uptake or fructolysis.

(c) *Experiments 3, 4, and 5: Effects of the Addition of Citrate, Ketoglutarate, or Malate*

Aliquots of four ejaculates, either controls or after storage at −79°C, were incubated in diluents containing fructose (6 mM), sodium lactate (0·1 mM), and with and without the inclusion of sodium citrate (9 mM). The addition of citrate was found to have no significant effect.

In a similar experiment, addition of sodium ketoglutarate (9 mM) also had no effect.

The only significant effect ($P<0·01$) of the addition of sodium malate (9 mM) to the incubation diluent was the decrease in fructose utilization by the recently collected semen from 1·59 μmoles per 10^8 cells per 3 hr in the absence of malate to 1·37 μmoles per 10^8 cells per 3 hr in the presence of malate. Lactate accumulation was unaffected.

In these three experiments freezing significantly decreased oxygen uptake, fructose oxidation, lactate oxidation, and lactate accumulation. As in experiments 1 and 2 there was a greater fall after freezing in the amount of $^{14}CO_2$ formed from [2-^{14}C]lactate than from [1-^{14}C]lactate.

(d) *Experiments 6, 7, and 8: Effects of the Addition of Succinate, Fumarate, and Oxaloacetate*

In experiment 6 aliquots of three ejaculates (2–3 $\times 10^8$ cells per flask) were incubated, either before or after freezing, in diluents containing fructose (6 mM) plus sodium lactate (0·1 mM), or fructose plus lactate plus sodium succinate (9 mM) or fumarate (9 mM) or oxaloacetate (9 mM). Mean results and standard errors of the means are given in Table 3.

Addition of oxaloacetate increased the oxygen uptake of fresh but not of frozen spermatozoa, and decreased the oxidation of fructose and lactate in both treatments. Thus the percentage of oxygen uptake due to fructose plus lactate oxidation was decreased with both fresh and stored cells ($P<0·05$). With recently collected spermatozoa the ratio of $^{14}CO_2$ from [2-^{14}C]lactate to $^{14}CO_2$ from [1-^{14}C]lactate was higher when oxaloacetate was present. Fructose utilization and lactate accumulation were both higher in the presence of oxaloacetate.

When succinate was added, recently ejaculated spermatozoa oxidized less fructose and lactate whereas there was no effect with stored cells. However, addition of succinate increased the oxygen uptake of both control and stored spermatozoa and oxidation of fructose plus lactate made up a smaller percentage of total oxygen utilized when succinate was present ($P<0·05$). Addition of fumarate had no effect.

The large increase in oxygen uptake on addition of succinate was further examined in two experiments. When frozen spermatozoa were thawed, washed, and incubated with fructose plus lactate plus 0, 1, 3, or 9 mM sodium succinate the oxygen uptakes were 1·30, 1·76, 2·47, and 3·85 μmoles per 10^8 cells per 3 hr respectively. There was no effect on motility. Inclusion of sodium succinate (3 mM) in the freezing diluents had no effect on the subsequent oxygen uptake or motility of the cells after thawing and washing.

(e) *Degree of Correlation between Parameters*

In experiments 1–6 a common treatment was the incubation of both recently ejaculated and stored spermatozoa in the presence of fructose (6 mM) and sodium lactate (0·1 mM). The parameters measured were: fraction of spermatozoa unstained, oxygen uptake per 10^8 cells and per 10^8 unstained cells, the fraction of the oxygen uptake not accounted for in oxidation of fructose and lactate (endogenous oxygen uptake), the $^{14}CO_2$ ratio (the $^{14}CO_2$ formed from [2-^{14}C]lactate expressed as a fraction of the $^{14}CO_2$ formed from [1-^{14}C]lactate), and lactate accumulated per 10^8 cells and per 10^8 unstained cells.

TABLE 3

EFFECT OF ADDITION OF SODIUM SUCCINATE OR FUMARATE OR OXALOACETATE (9 mM) ON THE METABOLISM OF RAM SPERMATOZOA BEFORE AND AFTER STORAGE (18 HR) AT −79°C

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

Treatment	Carbon Dioxide Produced from Labelled Carbon Atom of:				Oxygen Uptake	Fructose Utilized	Lactate Accumulated
	[U-^{14}C]-Fructose	[1-^{14}C]-Lactate	[2-^{14}C]-Lactate	[1,4-^{14}C]Succinate or Fumarate			
			Before Storage				
Control	1·53	0·094	0·081	—	2·29	1·55	1·97
+Succinate	1·29	0·084	0·076	0·222	2·72	1·41	1·76
+Fumarate	1·47	0·095	0·075	0·091	2·38	1·39	1·96
+Oxaloacetate	1·06	0·045	0·044	—	2·53	1·91	3·93
Standard error	0·036	0·0030			0·06	0·07	0·15
Degrees of freedom	6	14			24	12	12
			After Storage				
Control	0·081	0·026	0·014	—	0·27		0·04
+Succinate	0·061	0·023	0·015	0·038	1·49		0·10
+Fumarate	0·062	0·025	0·013	0·051	0·31		0·13
+Oxaloacetate	0·011	0·0011	0·0006	—	0·30		0·46
Standard error	0·016	0·0027			0·05		0·09
Degrees of freedom	6	14			6		6

There were no significant correlations between any parameter before and after freezing. When parameters measured in recently ejaculated semen were compared, only two significant correlations were observed. Oxygen uptake per 10^8 cells (mean 2·61 μmoles, S.E. 0·20) was significantly correlated ($r = 0·948$, $P < 0·001$, $n = 16$, $b = 0·647$) with lactate accumulated per 10^8 cells (mean 2·04 μmoles, S.E. 0·136). Oxygen uptake per 10^8 unstained cells (3·18 μmoles, S.E. 0·140) was significantly correlated ($r = 0·919$, $n = 14$, $P < 0·001$, $b = 0·326$) with lactate accumulated per 10^8 unstained cells (2·49 μmoles, S.E. 0·050). However, the recently ejaculated semen gave only a narrow range of values for cells unstained, $^{14}CO_2$ ratio, and endogenous oxygen uptake.

Most of the characteristics of semen incubated after storage were found to be correlated (Table 4). A similar pattern was observed when the values obtained after storage were expressed as a fraction of the values obtained with the same ejaculates before freezing (Table 5). However, in both these comparisons changes in endogenous oxygen uptake did not parallel changes in the other characteristics measured.

TABLE 4

PARALLELISM BETWEEN CHARACTERISTICS OF RAM SPERMATOZOA INCUBATED AFTER STORAGE AT −79°C

Mean values are expressed as μmoles per 10^8 spermatozoa or as fractions. The $^{14}CO_2$ ratio is the $^{14}CO_2$ formed from [2-^{14}C]lactate expressed as a fraction of that formed from [1-^{14}C]lactate. r = Sample correlation coefficient value; b = sample regression coefficient value; n = number of samples

	Mean	Standard Error	Value	Oxygen Uptake	Endogenous Oxygen Uptake Fraction	$^{14}CO_2$ Ratio	Lactate Accumulated	Lactate Accumulated/10^8 Unstained Cells
Mean				0·43	0·43	0·57	0·18	0·43
Standard error				0·085	0·037	0·033	0·067	0·135
Fraction of cells unstained	0·27	0·032	r	0·824***	0·423	0·722***	0·810***	
			b	2·171	0·486	0·729	1·513	
			n	19	17	19	16	
Oxygen uptake	0·43	0·085	r			0·720***	0·961***	
			b			0·276	0·642	
			n			19	16	
Endogenous oxygen uptake	0·43	0·037	r			0·367	0·444	0·506
			b			0·356	0·762	1·651
			n			17	14	14
$^{14}CO_2$ ratio	0·57	0·033	r				0·819***	0·842***
			b				1·640	3·396
			n				16	16
Oxygen uptake/10^8 unstained cells	1·51	0·160	r			0·370		0·896***
			b			0·075		0·708
			n			19		16

*** Significantly correlated at $P < 0·001$.

TABLE 5

PARALLELISM BETWEEN CHANGES IN CHARACTERISTICS OF SPERMATOZOA DUE TO FREEZING

Values are means of fractions obtained by expressing the data obtained after freezing as a fraction of that obtained before freezing. $^{14}CO_2$ ratio and r, b, and n are as for Table 4

	Mean	Standard Error	Value	Oxygen Uptake	Endogenous Oxygen Uptake	$^{14}CO_2$ Ratio	Lactate Accumulated	Lactate Accumulated/10^8 Unstained Cells
Mean				0·17	2·82	0·48	0·10	0·21
Standard error				0·034	0·202	0·041	0·031	0·054
Unstained cells (%)	0·28	0·034	r	0·772***	0·070	0·609**	0·699*	
			b	0·62	0·81	0·68	0·36	
			n	17	15	17	10	
Oxygen uptake	0·17	0·034	r			0·775***	0·948***	
			b			0·94	0·76	
			n			19	12	
Endogenous oxygen uptake	2·82	0·202	r			0·155	0·138	0·084
			b			0·01	0·01	0·01
			n			17	10	8
$^{14}CO_2$ ratio	0·48	0·041	r				0·872***	0·815**
			b				0·64	0·94
			n				16	10
Oxygen uptake/10^8 unstained cells	0·50	0·056	r			0·3515		0·897***
			b			0·24		0·50
			n			17		10

* Significantly correlated at $P < 0·02$. ** Significantly correlated at $P < 0·01$. *** Significantly correlated at $P < 0·001$.

IV. Discussion

In all experiments freezing resulted in considerable depression of both respiration and glycolysis. The significant correlations in the decreases due to freezing and between the various characteristics of thawed cells probably arise through several metabolic steps being damaged by a similar mechanism, which also results in membrane alterations such that the spermatozoa lose their selective permeability. Cellular damage to several tissues by freezing appears from various lines of evidence to be associated with alteration of the integrity of cell membranes (Lovelock 1957; Weiss and Armstrong 1960; Mann 1964; Doebbler and Rinfret 1965; Valeri, Bond, and McCallum 1966). The lack of correlation between endogenous oxygen uptake and other measurements is possibly due to the greater error when several measurements are used to get a final value. Also there would have been some residual glycerol present in the thawed semen, and metabolism of this will reduce the amount of fructose oxidized (Mann and White 1957; White 1957; Rao and Ehlers 1966).

The effects of freezing were not uniform, as shown by the greater depression in oxidation of [2-^{14}C]lactate than [1-^{14}C]lactate. This was expected as storage of ram and bull spermatozoa at 5°C gave a similar effect (O'Shea and Wales 1967*a*). As ram spermatozoa accumulate acetate when incubated with lactate (O'Shea and Wales 1968), this incomplete oxidation of lactate implies increased accumulation of acetate by thawed cells.

Mizuho, Niwa, and Soejima (1963) found only a slight depression of respiration of surviving bull and boar spermatozoa in semen preserved at −79°C, whereas the accumulation of lactic acid was much lower. There is also a larger depression in lactate accumulation than in oxygen uptake with ram semen (Table 5). These differences in degree of damage are probably related to the differing sensitivity of enzymes to freezing (Chilson, Costello, and Kaplan 1965).

The correlation between the percentage of unstained cells in thawed semen and their oxygen uptake and lactate accumulation indicates that the stained spermatozoa do not contribute to these metabolic measurements. It would then be valid to express metabolic data in terms of the number of unstained or "live" cells. The regression line for oxygen uptake passes through the origin but that for lactate accumulation does not. Thus there is no accumulation or even a net loss of lactate when less than 15% of the cells are unstained. This is because of the greater depression by freezing of fructolysis than of oxidation.

As reported for lactate accumulation by thawed bull semen (O'Dell and Almquist 1958; Mizuho, Niwa, and Soejima 1963), lactate accumulation and oxygen uptake of ram spermatozoa expressed per 10^8 unstained cells fell after freezing. Thus freezing impairs the metabolism of the surviving cells.

In general, recently ejaculated and thawed semen responded in a similar way to the addition of various substrates. The smaller rate of increase in oxidation of lactate with lactate addition after storage is due to there being little fructose oxidation to be replaced. In agreement with O'Shea and Wales (1966) the presence of small amounts of sodium lactate (5 mM) had no effect on fructolysis, while large amounts (25 mM) depressed fructolysis as reported by Amir and Schindler (1967). However, at pH 7·2 and with 5 and 25 mM sodium lactate there was no effect of lactate addition

on oxygen uptake. Amir and Schindler (1967) observed that at pH 6·7, 44 mM lactate depressed oxygen uptake, whereas at pH 6·0, 11 mM was sufficient to decrease respiration. This interaction and the small differences from the present report emphasizes that depression of cell metabolism by addition of lactate occurs more readily as the pH is lowered.

As previously reported for fresh semen (O'Shea and Wales 1966) inclusion of acetate did not affect oxygen uptake or fructolysis. The lack of effect of citrate disagrees with the decrease in lactate accumulation previously reported (O'Shea and Wales 1966). This may have been due to the inclusion of lactate in the diluents in the present paper, but the results in Table 1 seem to preclude such low levels of lactate from having any effect on fructolysis.

From the results of experiments 6, 7, and 8 the increased oxygen uptake of thawed cells on addition of succinate is unrelated to normal metabolism and to motility. A similar effect is seen with cold-shocked or detergent-treated spermatozoa, and this apparent activation of succinic dehydrogenase is a manifestation of cellular and metabolic disorganization (Mann 1964).

Oxaloacetate is readily utilized by ram and bull spermatozoa (Humphrey and Mann 1949; Graves and Salisbury 1966), and in the present paper has been shown to cause a marked reduction in the oxidation of fructose and lactate. Flipse (1967) has shown that there is a rate-limiting step between citrate and α-ketoglutarate in the citric acid cycle in bull spermatozoa. Freely available oxaloacetate would thus allow formation of ample citrate (Humphrey and Mann 1949), but despite the increase in fructolysis on addition of oxaloacetate to the incubation diluent there was decreased entry of fructose and lactate into the citric acid cycle. This, together with incomplete oxidation of lactate, as shown by the difference between the oxidation of [1-^{14}C]- and [2-^{14}C]lactate, and the finding that CO_2 can be fixed by spermatozoa with pyruvate as substrate (Mounib and Eisan 1967; O'Shea and Wales 1967*b*), shows that lactate, whether added to the incubation medium or formed by glycolysis of hexose, does not merely enter the citric acid cycle.

V. Acknowledgments

The author is indebted to Professor C. W. Emmens and Dr. R. G. Wales for their interest and criticism. This work was aided by grants from the Rural Credits Development Fund of the Commonwealth Bank of Australia and the Australian Wool Board.

VI. References

Amir, D., and Schindler, H. (1967).—Influence of lactate on the rates of respiration in ram spermatozoa. *J. Reprod. Fert.* **14**, 121.

Chilson, O. P., Costello, L. A., and Kaplan, N. O. (1965).—Effects of freezing on enzymes. *Fedn Proc. Fedn Am. Socs exp. Biol.* **24** (Suppl. 15), S-55.

Doebbler, G. F., and Rinfret, A. P. (1965).—Rapid freezing and thawing of blood. Physical and chemical considerations of injury and protection. *Cryobiology* **1**, 205.

Duncan, D. B. (1955).—Multiple range and multiple *F* tests. *Biometrics* **11**, 1.

Flipse, R. J. (1967).—Metabolism of bovine semen. XV. Rate-limiting reactions in the citric acid cycle. *J. Dairy Sci.* **50**, 381.

GRAVES, C. N., and SALISBURY, G. W. (1966).—The metabolism of carbohydrates and other compounds by epididymal-like bovine spermatozoa. *J. Reprod. Fert.* **12**, 213.

HENDLER, R. W. (1959).—Self absorption correction for carbon-14. *Science, N.Y.* **130**, 772.

HUMPHREY, G. F., and MANN, T. (1949).—Studies on the metabolism of semen. 5. Citric acid in semen. *Biochem. J.* **44**, 97.

LOVELOCK, J. E. (1957).—The denaturation of lipid–protein complexes as a cause of damage by freezing. *Proc. R. Soc.* B **147**, 427.

MANN, T. (1964).—"The Biochemistry of Semen and of the Male Reproductive Tract." (Methuen & Co., Ltd.: London.)

MANN, T., and WHITE, I. G. (1957).—Glycerol metabolism by spermatozoa. *Biochem. J.* **65**, 634.

MIZUHO, A., NIWA, T., and SOEJIMA, A. (1963).—Basic studies on the preservation of semen of farm animals. VI. The effect of freezing on the metabolic activity of spermatozoa. *Bull. natn. Inst. Anim. Ind., Chiba* **9**, 99.

MOUNIB, M. S., and EISAN, J. S. (1967).—Fixation of carbon dioxide by cod sperm. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 645.

O'DELL, W. T., and ALMQUIST, J. O. (1958).—Freezing bovine semen. IV. Effect of freezing on the metabolic activity of bovine spermatozoa during and after storage at −79°C. *J. Dairy Sci.* **41**, 1792.

O'SHEA, T. (1969).—Metabolism of semen after freezing. I. Effects of washing, skim-milk solids, and potassium and magnesium ions on the metabolism of thawed ram and bull spermatozoa. *Aust. J. biol. Sci.* **22**, 721.

O'SHEA, T., and WALES, R. G. (1965).—Metabolism of sorbitol and fructose by ram spermatozoa. *J. Reprod. Fert.* **10**, 353.

O'SHEA, T., and WALES, R. G. (1966).—The aerobic metabolism of ram spermatozoa. *J. Reprod. Fert.* **11**, 263.

O'SHEA, T., and WALES, R. G. (1967*a*).—The metabolism of ram, bull, dog, and rabbit spermatozoa after cooling to 5°C. *Aust. J. biol. Sci.* **20**, 447.

O'SHEA, T., and WALES, R. G. (1967*b*).—Fixation of carbon dioxide by ram spermatozoa. *J. Reprod. Fert.* **14**, 333.

O'SHEA, T., and WALES, R. G. (1968).—Metabolism of [1-^{14}C]sodium lactate and [2-^{14}C]sodium lactate by ram spermatozoa. *J. Reprod. Fert.* **15**, 337.

PATTERSON, M. W., and GREENE, R. C. (1965).—Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Analyt. Chem.* **37**, 854.

RAO, CH. V., and EHLERS, M. H. (1966).—Glucose, glycerol and glycine metabolism in bovine spermatozoa and certain interactions. *J. Dairy Sci.* **49**, 725.

SNEDECOR, G. W. (1956).—"Statistical Methods." 5th Edn. (Iowa State College Press: Ames.)

VALERI, C. R., BOND, J. C., and MCCALLUM, L. E. (1966).—Relationships between metabolic state and (1) *in vivo* survival and (2) density distribution of previously frozen human erythrocytes. *Transfusion* **6**, 543.

WEISS, L., and ARMSTRONG, J. A. (1960).—Structural changes in mammalian cells associated with cooling to −79°C. *Biophys. Biochem. Cytol.* **7**, 673.

WHITE, I. G. (1957).—Metabolism of glycerol and similar compounds by bull spermatozoa. *Am. J. Physiol.* **189**, 307.