METABOLISM OF [1-¹⁴C]SODIUM LACTATE AND [2-¹⁴C]SODIUM LACTATE BY RAM SPERMATOOZOA

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Summary. The metabolism of specifically labelled lactate by ram spermatozoa was studied under various experimental conditions. Increasing the rate at which diluted ram semen was cooled to 5° C caused a greater decrease in the formation of labelled carbon dioxide from [2-¹⁴C]lactate than from [1-¹⁴C]lactate. Changes in the amount of lactate present did not affect the relative amounts of carbon dioxide formed from carbon atoms one and two of lactate.

Washing ram spermatozoa, that had been recently ejaculated, decreased the amount of labelled carbon dioxide formed from [2-¹⁴C]lactate relative to that formed from [1-¹⁴C]lactate, whereas the reverse effect occurred on washing spermatozoa that had been stored in test tubes at 37° C for 3 hr.

When washed ram spermatozoa were incubated with lactate as substrate, labelled acetic acid accumulated from [2-¹⁴C]lactate but not from [1-¹⁴C]lactate, both in the absence and in the presence of added potassium chloride (1 mM) and magnesium chloride (2 mM).

INTRODUCTION

Ram spermatozoa form more carbon dioxide from carbon-one than from carbon-two of lactate and this difference has been found to increase on cooling and on storage at 5° C (O'Shea & Wales, 1967). Thus part of the lactate forming acetyl CoA does not undergo complete oxidation in the citric acid cycle and some product other than carbon dioxide must accumulate.

In the present paper, the effects of a number of changes in environmental conditions on the relative amounts of carbon dioxide from carbons one and two of lactate are investigated. As potassium and magnesium are important in the metabolism of ram spermatozoa, whether recently collected or stored (Lardy & Phillips, 1943; Blackshaw, 1953; White, 1953a, b; Dott & White, 1964; Wallace & Wales, 1964; Wales & O'Shea, 1966; O'Shea & Wales, 1967), particular attention is paid to the effect of these ions on the ratio of carbon dioxide formed from carbons-1 and 2 of lactate during metabolism. The accumulation of carboxylic acids from specifically labelled lactate is also examined.

MATERIALS AND METHODS

General

Semen, diluents, incubation in Warburg flasks, analytical techniques and statistical methods have been described (O'Shea & Wales, 1966). The percentage of spermatozoa unstained was estimated using congo red nigrosin (Blackshaw, 1958) and motility was given a score between 4 and 0 (Emmens, 1947). Data expressed as percentages were converted to angles before statistical analysis was performed.

In Experiment 1, normal cooling to 5° C took 2 to 3 hr and was carried out by surrounding the test tubes containing diluted semen with a water jacket before cooling was begun (O'Shea & Wales, 1964). Fast cooling was carried out by placing the test tubes in a cold room without a water jacket, and spermatozoa were cold-shocked by plunging the test tubes into water at 5° C.

Isolation of organic acids

The technique of partition chromatography on silicic acid was adapted for the collection of radio-active eluents and their direct assay by liquid scintillation techniques.

For the column 6 g of silicic acid (100 Mesh, Mallinckrodt Chemical Works) was triturated with 4 ml of 0.5 N H₂SO₄, made into a slurry with acidified hexane (0.5 N H₂SO₄) and transferred to a glass chromatographic tube of

TEXT-FIG. 1. The separation of organic acids by partition chromatography on silicic acid with elution by hexane:butanol.

10 mm internal diameter and 30 cm long. The sample to be analysed (approximately 0.2 ml) was acidified with 5 N H₂SO₄, triturated with 0.5 g silicic acid and transferred into the top of a prepared column.

Hexane:butanol solvents (Neish, 1949) were used and a mixing device was employed to increase progressively the concentration of butanol in the hexane applied to the column (see Donaldson, Tulane & Marshall, 1952). The lower reservoir, with a volume of 150 ml, contained hexane equilibrated with

0.5 N H_2SO_4 and a magnetic stirrer. The solvent blending was accomplished by delivering 100 ml acidified hexane:butanol (1:1) followed by 100 ml acidified hexane:butanol (1:3), about 3 cm below the surface of the hexane. Where necessary, after 200 ml of eluent had been collected, the resultant mixture in the lower reservoir was added directly to the liquid above the column. For scintillation counting the fractions (3.4 ml) were added to 6 ml of xylene containing 0.4% (w/v) 2,5-diphenyl-oxazole (PPO) and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Dimethyl POPOP), obtained from the Packard Instrument Company.

Radio-active peaks were recovered and re-chromatographed in the presence of known labelled compounds using a constant solvent system (Bulen, Varner & Burrell, 1952). The substance responsible for the radio-active peak tentatively identified as acetate, was volatile on steam distillation (95% recovery) and ran with acetate when re-chromatographed with the addition of authentic labelled acetate or formate using hexane:n-butanol (93:7). The acetate peak was not observed if aliquots were acidified before drying at room temperature.

Using the system described, isotopically labelled acids, singly, in pairs and in mixtures, were placed in columns to determine the order in which they were eluted (Text-fig. 1).

RESULTS

Aliquots of ram semen, diluted with 9 volumes of a diluent containing fructose (4 mM) and casein (4 g/100 ml w/v) were cooled to 5° C at three different rates and re-warmed to 37° C about 4 hr after semen collection. The percentage of spermatozoa unstained and their respiration were compared with those of control aliquots, stained or incubated shortly after collection. To obtain a similar concentration of fructose in the suspension cooled to 5° C as in the controls, more fructose (3 $\mu\text{mole}/\text{flask}$) was added before incubation of the treated aliquots (0.8 ml) in small Warburg flasks. Mean results (Table 1) for four ejaculates (1.8 to 3.1×10^8 cells/flask) show a decrease in the $^{14}\text{CO}_2$ ratio ($P < 0.05$) and in the percentage of spermatozoa unstained ($P < 0.01$) with increasing rate of cooling. The decrease in oxygen uptake with increasing rate of cooling became less as the cooling rate increased ($P < 0.05$).

TABLE 1
EFFECT OF RATE OF COOLING TO 5° C ON RAM SPERMATOZOA

Treatment	Oxygen uptake ($\mu\text{mole}/10^8$ sperm.)	$^{14}\text{CO}_2$ ratio	Spermatozoa unstained (%)
Control	3.35	94.4	93
Normal cooling	1.98	77.6	71
Fast cooling	1.21	64.6	60
Cold shock	0.87	58.8	37
S.E.M. (d.f.)	0.28 (9)	6.96 (9)	2.5 (9)

Values are the means for four ejaculates. The $^{14}\text{CO}_2$ ratio is the $^{14}\text{CO}_2$ formed from $[2\text{-}^{14}\text{C}]\text{lactate}$ expressed as a percentage of the $^{14}\text{CO}_2$ formed from $[1\text{-}^{14}\text{C}]\text{lactate}$.

TABLE 2
EFFECT OF pH ON WASHED RAM SPERMATOZOA WITH LACTATE AS SOLE
SUBSTRATE

pH	Oxygen uptake	CO ₂ formed from carbon atom labelled		¹⁴ CO ₂ ratio	Motility
		[1- ¹⁴ C]lactate	[2- ¹⁴ C]lactate		
6.1	1.78	0.69	0.53	76	2.7
6.9	2.55	0.84	0.76	90	2.8
7.6	2.81	0.80	0.73	92	1.8
S.E.M. (d.f.)	0.12 (6)	0.07 (6)		3.37 (6)	0.14 (12)

Values are the means for four ejaculates and the metabolic data are expressed as $\mu\text{mole}/10^8$ spermatozoa over the experimental period (3 hr). The ¹⁴CO₂ ratio is the ¹⁴CO₂ formed from [2-¹⁴C]lactate expressed as a percentage of the ¹⁴CO₂ formed from [1-¹⁴C]lactate.

In the next experiment spermatozoa were washed and incubated in diluents containing sodium lactate (12 mM), potassium chloride (1 mM), magnesium chloride (2 mM) and 20 mM mono- and di-sodium phosphate buffer at three pH values (6, 7 or 8). After the addition of 1 volume of semen to 9 volumes of each diluent the mean pH in the three suspensions was 6.1, 6.9 and 7.6. Aliquots (0.9 ml) of four ejaculates (1.4 to 4.3×10^8 cells/flask) were incubated in Warburg flasks (5 ml) and the results are presented in Table 2. There was a decreased oxygen uptake at pH 6.1 ($P < 0.01$) and lowered motility at pH 7.6 ($P < 0.01$). However (due to variation between ejaculates), there was no significant effect of pH on the production of carbon dioxide. Overall, there was more carbon dioxide formed from carbon atom one than from carbon atom two of lactate ($P < 0.01$).

The metabolism of aliquots of semen diluted with 6 volumes of a potassium- and magnesium-free diluent containing fructose (6 mM) and stored in test tubes

TABLE 3
EFFECT OF WASHING AND OF STORAGE AT 37° C (3 HR) ON THE OXIDATIVE
METABOLISM OF RAM SPERMATOZOA INCUBATED WITH FRUCTOSE (6
 $\mu\text{MOLE/FLASK}$) AND LACTATE

Treatment	Washing	Lactate ($\mu\text{mole/flask}$)	Oxygen uptake ($\mu\text{mole}/10^8$ cells)	¹⁴ CO ₂ ratio
Control	—	4	1.82	77.4
		12	1.76	85.4
	+	4	1.74	69.6
		12	1.67	65.9
Stored at 37° C	—	4	0.65	35.8
		12	0.68	35.3
	+	4	0.70	43.5
		12	0.74	46.7
S.E.M. (d.f.)			0.018 (14)	3.17 (14)

Values are the means for three ejaculates. The ¹⁴CO₂ ratio is the ¹⁴CO₂ formed from [2-¹⁴C]lactate expressed as a percentage of the ¹⁴CO₂ formed from [1-¹⁴C]lactate.

at 37° C for 3 hr, was compared with that of aliquots incubated shortly after collection. To help determine whether any effects of storage were due to changes in the cells themselves or to changes in the composition of the seminal plasma, aliquots washed immediately before incubation were compared with unwashed aliquots. Aliquots of diluted semen (0.9 ml) were incubated in Warburg flasks

TABLE 4

OXIDATION OF LACTATE AND ACETATE BY WASHED RAM SPERMATOZOA WITH AND WITHOUT THE ADDITION OF K^+ (1 mM) AND Mg^{2+} (2 mM)

Substrate (μ mole/flask)		$K^+ + Mg^{2+}$	Position of ^{14}C label	Oxygen uptake	CO_2 formed from carbon position labelled		$^{14}CO_2$ ratios	
Lactate	Acetate				Lactate	Acetate	Lactate	Acetate
30	0	—	1	1.48	0.58	—	64	—
			2	1.41	0.37	—		
		+	1	2.87	1.08	—	98	—
			2	2.82	0.96	—		
15	22.5	—	1	1.62	0.58	0.34	23	82
			2	1.55	0.13	0.28		
		+	1	2.86	0.85	0.67	39	82
			2	2.82	0.33	0.55		
0	45	—	1	1.18	—	0.55	—	77
			2	1.23	—	0.41		
		+	1	2.71	—	1.39	—	78
			2	2.72	—	1.09		

Values are mean results for four ejaculates and are expressed as μ mole/ 10^8 spermatozoa over the experimental period (3 hr). The $^{14}CO_2$ ratio is the $^{14}CO_2$ formed from $[2-^{14}C]$ lactate or $[2-^{14}C]$ -acetate expressed as a percentage of the $^{14}CO_2$ formed from $[1-^{14}C]$ lactate or $[1-^{14}C]$ acetate.

SUMMARY OF THE ANALYSES OF VARIANCE

Source of variation	O_2 uptake		CO_2 formed		
	d.f.	Variance ratio	d.f.	Variance ratios	
				Lactate	Acetate
A: Substrate differences	2	5.57**	1	53.30**	65.91**
B: Addition of $K^+ + Mg^{2+}$	1	473.64**	1	108.02**	115.63**
C: Carbon atom labelled	1	0.21	1	74.50**	9.79**
Interactions					
A $\times$ B	2	1.38	1	17.42**	21.84**
A $\times$ C	2	0.18	1	18.55**	1.71
Other	3	0.02	2	0.56	0.83
Ejaculate differences	3	89.34**	3	23.09**	13.80**
Ejaculate interactions (error)	33	0.0486	21	0.0113	0.0187

** $P < 0.01$.

of 5 ml volume and the washed aliquots were reconstituted in an appropriate volume of diluent to ensure that sperm numbers in the unwashed and washed samples were similar (mean 2.2×10^8 cells/flask). Shortly before incubation small amounts of diluents containing appropriate quantities of fructose and/or lactate were added to the samples so that flasks containing 6 μ mole fructose plus 4 μ mole lactate were compared with flasks containing 6 μ mole fructose

A*

plus 12 μmole lactate. The semen at incubation had been diluted approximately 1:9. The results of four replicates (Table 3) show an interaction of the effects of washing and of storing at 37° C on the ratio of the amount of carbon dioxide formed from carbon atom two of lactate to the amount of carbon dioxide formed from carbon atom one. With fresh semen the ratio was lower after washing. Storage at 37° C resulted ($P < 0.01$) in greater decreases in the $^{14}\text{CO}_2$

TABLE 5
EFFECTS OF KCl (1 mM) AND MgCl_2 (2 mM) ON THE OXIDATION OF LACTATE BY
WASHED RAM SPERMATOZOA

Buffer	$\text{K}^+ + \text{Mg}^{2+}$	Position of ^{14}C label	O_2 uptake (μmole)	CO_2 formed from carbon position labelled (μmole)	^{14}C accumulated as acetate (mpc)
Phosphate	—	1	1.79	0.62	8.56
		2	1.77	0.45	
	+	1	2.79	0.76	7.86
		2	2.84	0.65	
Tris	—	1	2.42	0.87	11.58
		2	2.35	0.68	
	+	1	2.92	0.90	7.56
		2	2.87	0.76	

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

SUMMARY OF ANALYSES OF VARIANCE

Source of variation	d.f.	Variance ratios		Source of variation	d.f.	Variance ratio (Acetate accumulated)
		O_2 uptake	CO_2 formed			
A: Difference between buffers	1	7.35*	28.22**	A: Difference between buffers	1	0.73
B: Addition of $\text{K}^+ + \text{Mg}^{2+}$	1	38.04**	10.37**	B: Addition of $\text{K}^+ + \text{Mg}^{2+}$	1	2.21
C: Carbon position labelled	1	0.03	19.31**			
Interactions				Interaction		
A $\times$ B	1	4.42*	2.69	A $\times$ B	1	1.11
Other	3	0.04	0.22			
Ejaculate differences	3	13.80**	21.53**	Ejaculate differences	3	1.76
Ejaculate interactions (error)	21	0.1253	0.0099	Ejaculate interactions (error)	9	9.985

* $P < 0.05$. ** $P < 0.01$.

ratio of unwashed spermatozoa (57% decrease) than of washed spermatozoa (30% decrease) so that, after storage at 37° C, the $^{14}\text{CO}_2$ ratio was higher for washed than for unwashed spermatozoa. Storage at 37° C greatly depressed oxygen uptake ($P < 0.01$). There were no effects of changing the concentration of substrate.

Aliquots of four ejaculates (3.1 to 8.5×10^8 cells/flask) were incubated in double side-arm Warburg flasks (volume 15 to 30 ml) with diluents containing lactate (30 μmole /flask), lactate (15 μmole /flask) plus acetate (22.5 μmole /

flask), or acetate alone (45 μ mole/flask) either with or without the addition of potassium and magnesium. After temperature equilibration, 0.3-ml aliquots of washed spermatozoal suspension were added from a side arm to 2.7 ml of incubation diluent in the main compartment and incubated at 37° C for 3 hr. The results and statistical analysis are given in Table 4.

The effects of the inclusion of potassium (1 mM) and magnesium (2 mM) chlorides in the diluent on the metabolism of lactate by washed ram spermatozoa were studied further. Using the basic diluent containing sodium lactate (6 mM) and a diluent containing trishydroxymethylaminomethane-HCl (tris) buffer (20 mM), sodium chloride (128 mM), and sodium lactate (6 mM), spermatozoa were incubated at 37° C for 3 hr in small Warburg flasks with and without the addition of potassium plus magnesium. After incubation, metabolism was terminated by adding 0.8 ml of the flask contents to 3.6 ml of ethanol containing 0.1 ml of 2 N NaOH which was placed in a water bath (70° C) for 5 min. After centrifuging, the carboxylic acids present in the supernatant were separated by silicic acid chromatography. The [1-¹⁴C]lactate and [2-¹⁴C]lactate were purified by silicic acid chromatography before use and aliquots, incubated in the absence of semen, were used as controls in the silicic acid chromatograms. The mean results for four ejaculates (2.0 to 4.2 $\times 10^8$ cells/flask) are shown in Table 5. Accumulation of acetate and decrease in lactate were the only changes observed in the silicic acid chromatograms. Accumulation of acetate was not observed using [1-¹⁴C]lactate and the treatments had no significant effect on the accumulation of labelled acetate from [2-¹⁴C]lactate.

DISCUSSION

The present experiments show that a number of treatments can influence the relative production of carbon dioxide from carbons one and two of lactate. In general, unfavourable environmental influences decrease the oxidation of carbon two of lactate compared with that of carbon one, and this alteration in lactate metabolism would appear to correspond to the degree of cell damage. Thus, increasing the rate at which the cells are cooled to 5° C decreases the ratio of carbon dioxide formed from carbon two to that formed from carbon one and this change closely parallels the effects of cooling on the percentage of spermatozoa stained by supravital dyes. Ageing of the cells by incubation at 37° C for 3 hr also decreases the above ratio. In buffered suspensions of spermatozoa the accumulation of lactate during cooling and storage would not be expected to change the pH sufficiently to cause the alteration in metabolism of lactate noted in previous reports (O'Shea & Wales, 1966, 1967).

The present experiments indicate that decreases in the ratio:

$$\frac{{}^{14}\text{CO}_2 \text{ from } [2\text{-}^{14}\text{C}]\text{lactate}}{{}^{14}\text{CO}_2 \text{ from } [1\text{-}^{14}\text{C}]\text{lactate}}$$

are related to changes in the incubation diluent as well as to changes in the cell itself. Hence, removal of seminal plasma and replacement by buffered saline decreases the ratio. This is probably due to the removal of potassium and magnesium ions as their addition to washed suspensions returned the ratio almost to

unity. Thus, the use of $[1-^{14}\text{C}]$ lactate probably over-estimates lactate oxidation by suspensions of washed spermatozoa where K^+ and Mg^{2+} are not added. With unwashed suspensions, the ratio is near unity and the use of $[1-^{14}\text{C}]$ lactate does not bias the estimates.

Another important environmental influence on the ratio is the presence in the medium of alternate substrates which, with lactate, form a metabolic pool for entry into the citric acid cycle. The substitution of acetate for some of the lactate in the incubation diluent greatly suppresses the oxidation of carbon two of lactate. The present experiments have shown that acetate can accumulate during incubation with lactate as substrate and such an accumulation probably accounts for some of the decrease in the ratio that occurs when diluted semen is kept at 37°C for 3 hr before oxidation is measured. This is not the only cause of the alteration in lactate metabolism under these conditions as cells kept at 37°C for 3 hr and washed before incubation still show a decreased capacity to oxidize carbon two of lactate.

Since spermatozoa oxidize lactate and acetate to carbon dioxide through the Krebs' tricarboxylic acid cycle (Salisbury & Lodge, 1962; Scott, White & Annison, 1962; Flipse, 1964; Mohri, Mohri & Ernster, 1965), the accumulation of labelled acetate from $[2-^{14}\text{C}]$ lactate and not from $[1-^{14}\text{C}]$ lactate is explicable. Because of the increased rate of entry of acetate into the citric acid cycle and the effect on the citric acid cycle caused by potassium and magnesium (Beinert, Green, Hele, Hift, Von Korff & Ramakrishnan, 1953; Von Korff, 1953; Krebs & Lowenstein, 1960), it was anticipated that there would be less acetate accumulated when these ions were added. Although this did not occur, inspection of the data reveals that this effect occurred in the two more dilute ejaculates but not in the two more dense ejaculates. Thus, a low sperm density may be necessary to show this effect consistently.

According to Krebs & Lowenstein (1960), the intermediates of the tricarboxylic acid cycle do not normally accumulate to appreciable levels in respiring cells. This would appear to be the case for spermatozoa as only acetate accumulation was observed on chromatography.

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