THE AEROBIC METABOLISM OF RAM SPERMATOZOA

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Summary. The relative contributions of fructose, lactate, acetate, citrate and inositol to the aerobic metabolism of ram spermatozoa have been examined in various combinations of these substrates. By using isotopically labelled compounds it was shown that fructose and lactate were important substrates oxidized by ram spermatozoa incubated in diluents containing fructose and low levels of other metabolites. Although the addition of sodium lactate to a spermatozoal suspension caused no change in the amount of fructose converted to lactate, there was a reduction in the amount of fructose oxidized to carbon dioxide.

At the levels of acetate and citrate occurring in semen, their effect on the oxidation of fructose by ram spermatozoa was small. Higher concentrations of these compounds, however, caused a decrease in fructose oxidation. The addition of citrate and acetate to a fructose-containing diluent did not affect the amount of fructose utilized by ram spermatozoa. While acetate had no effect on the accumulation of lactate from fructose, the presence of the high levels of citrate decreased the amount of lactate produced.

Thus fructose and lactate are the main metabolites for unwashed ram semen incubated under aerobic conditions in a diluent containing fructose. In these circumstances, a quantitative measure of the oxidation of these substrates, coupled with an estimation of fructolysis, should give a good measure of the metabolism of the cell.

INTRODUCTION

A number of substrates occur in the seminal plasma of the ram (Mann, 1964) and, during aerobic incubations of spermatozoa in unwashed suspensions, each substrate may be oxidized to a varying extent. Little is known of the contribution of these native substrates to the overall metabolism of unwashed cells incubated in the presence of an added substrate such as fructose. Wallace & Wales (1964), using a fructose-containing medium, have shown that washing ram spermatozoa free of seminal plasma causes a reduction in the oxidation of substrates other than fructose. This is probably related to the removal of alternative substrates during washing. In the present paper, the metabolism of various combinations of substrates has been investigated, especially in relation to effects on fructose metabolism.

MATERIALS AND METHODS

Semen and diluents

Ram semen was collected by electrical stimulation and only apparently normal ejaculates with good initial motility were used. Washed spermatozoal suspensions were obtained by diluting the semen with 10 vols. of basic diluent and centrifuging at 400 *g* for 7 min. The supernatant was replaced with an equal volume of diluent and the spermatozoa dispersed and re-centrifuged. After removing the second supernatant, the spermatozoa were diluted to the required volume.

The basic diluent used consisted of 20 mM-mono- and di-sodium phosphate buffer (pH 7.0), 127 mM-sodium chloride, 30 mg% penicillin and 50 mg% streptomycin. When substrates were included in the diluent, isotonicity was maintained at 308 milliosmoles/litre by decreasing the sodium chloride content. To measure substrate oxidation, isotopically labelled substrate was added to the flasks as carrier-free isotope in 0.1 ml of 0.9% sodium chloride. Where more than one substrate was present in a diluent, duplicate or triplicate Warburg flasks were prepared for each treatment and each flask contained one labelled substrate as well as the combination of unlabelled substrates. By assay of the trapped carbon dioxide in each flask, the contribution of each substrate to the total oxygen uptake could be measured. The usual level of isotope added to a flask was 50 m μ C D-U-¹⁴C-fructose, 60 m μ C DL-1-¹⁴C-sodium lactate, 50 m μ C 1-¹⁴C-sodium acetate, 100 m μ C U-¹⁴C-myo-inositol or 100 m μ C 1,5-¹⁴C-sodium citrate. The labelled compounds were obtained from the Radiochemical Centre, Amersham.

Incubation of spermatozoa

The experiments were carried out at 37° C in Warburg flasks containing carbon dioxide-free KOH (20% w/v) in the centre well. In the first two experiments, and in the experiment with inositol, aliquots of semen were diluted in 15 vols of the appropriate diluents and 0.8 ml incubated in 5 ml Warburg flasks. The other experiments were carried out in double side-arm Warburg flasks of 16 to 24 ml volume, containing 2.5 ml of substrate diluent and 0.1 ml of a labelled compound in the main compartment. The spermatozoal suspension (0.3 ml) was added to the side-arm and, after temperature equilibration, was mixed with the substrate diluent. The incubation was terminated by adding 0.1 ml of 6 N-H₂SO₄ from the other side-arm.

Analytical methods

Protein-free filtrates obtained by precipitation with equal volumes of 5% w/v ZnSO₄.7H₂O and 0.3 N-Ba(OH)₂, were used to determine the initial and final levels of fructose and L-lactate. Fructose was determined by the method of Mann (1948) as modified by White (1959) and lactate by an enzymatic method outlined by Barker & Britton (1957). The ¹⁴CO₂ was precipitated as Ba¹⁴CO₃ and assayed for radioactivity as described by Annison & White (1961) and corrected for self-absorption by the method of Hendler (1959). Spermatozoal counts were made in a haemocytometer. The method for the

measurement of levels of intracellular constituents is described elsewhere (Wales & O'Shea, 1966a).

Statistical analysis

The significance of the results has been assessed by analysis of variance, or by paired *t*-test where more appropriate. In the experiments where duplicate samples were tested, the variance between duplicates was used as error, except where the ejaculate interaction variance was significantly greater than the variance between duplicates. In the smaller experiments, pooled ejaculate interactions were used as error. In order to conserve space, the statistical analyses are not presented in the tables. Instead, the standard errors of the printed means (S.E._m) calculated from the error variance are given, with the associated degrees of freedom in parenthesis. The statistical significance of the results is quoted in the text.

RESULTS

The effect of increasing the level of lactic acid in the presence of a constant level of added fructose on the metabolism of unwashed ram semen was investigated in the first experiment. The diluents contained fructose and either no lactic acid or increasing levels of lactic acid. Added fructose plus the fructose contributed by the seminal plasma gave a mean initial fructose content of

TABLE 1
METABOLISM OF RAM SEMEN IN THE PRESENCE OF FRUCTOSE (4.4 μ MOLE/FLASK) AND VARIOUS LEVELS OF ADDED LACTIC ACID

<i>Molar ratio of fructose : lactate</i>	<i>O₂ uptake</i>	<i>Fructose oxidized</i>	<i>Lactate oxidized</i>	<i>Fructose utilized</i>	<i>Lactate accumulated</i>
1 : 0.5 to 0.9	1.24	0.078	0.249	0.82	1.15
1 : 0.9 to 1.3	1.21	0.062	0.280	0.75	1.02
1 : 1.8 to 2.3	1.10	0.029	0.326	0.62	0.84
1 : 2.6 to 3.3	1.04	0.016	0.365	0.45	0.37
S.E. _m	0.023 (16)	0.004 (9)	0.014 (9)	0.048 (9)	0.108 (9)

All values are expressed as μ mole/10⁸ spermatozoa over the experimental period (2 hr) and are the means for four ejaculates.

4.4 μ mole/flask. Fructose to lactate ratios, calculated from assays of the diluted semen, are given in Table 1. The results for four ejaculates (3 to 7 $\times$ 10⁸ cells/flask) showed that there was a significant linear fall in oxygen uptake, fructose oxidation, fructose utilization and lactate accumulation when the concentration of lactic acid was increased ($P < 0.01$). The decrease in fructose oxidation was relatively greater than the decrease in other parameters measured, and there was a significant linear increase in lactate oxidation with the addition of lactic acid ($P < 0.01$).

Under the conditions of the above experiment, the addition of 10 mM-lactic acid to diluted semen gave an average depression in pH of 0.5 units. To differentiate between the effects of pH and those of lactate addition, the effects

D*

of added lactic acid (10 mM) and sodium lactate (10 mM) were compared with a control containing fructose as sole substrate. The mean initial fructose level was 5 $\mu\text{mole/flask}$ and the results for four ejaculates (2 to 6×10^8 cells/flask) are given in Table 2. The addition of sodium lactate had no effect on oxygen uptake, fructose utilization or lactate accumulation. There was a highly significant decrease in fructose oxidation, with a concomitant increase in lactate oxidation on addition of both lactic acid and sodium lactate. The addition of lactic acid caused decreases in oxygen uptake, fructose utilization and lactate accumulation.

To examine whether changes in the concentration of acetate altered the metabolism of fructose, four ejaculates of washed ram spermatozoa (4 to 8×10^8 cells/flask) were incubated in the presence of a constant level of fructose (15 $\mu\text{mole/flask}$) and either no acetate or increasing levels of sodium acetate

TABLE 2

METABOLISM OF RAM SEMEN IN THE PRESENCE OF ADDED FRUCTOSE (5 $\mu\text{mole/flask}$) WITH AND WITHOUT THE ADDITION OF SODIUM LACTATE OR LACTIC ACID (8 $\mu\text{mole/flask}$)

Substrate added	O ₂ uptake	Fructose oxidized	Lactate oxidized	Fructose utilized	Lactate accumulated
A. Fructose	1.23	0.087†	0.237†	0.70	0.98
B. Fructose+ Na lactate	1.24	0.023	0.417	0.67	0.76
C. Fructose+ lactic acid	0.97*	0.009	0.405	0.28*	0.37*
S.E. _m	0.055 (12)	0.011 (6)	0.022 (6)	0.061 (6)	0.097 (6)

All values are expressed as $\mu\text{mole}/10^8$ spermatozoa over the experimental period (2 hr) and are the means for four ejaculates.

* C significantly different from A and B; $P < 0.01$.

† A significantly different from B and C; $P < 0.01$.

(2.5 to 15 $\mu\text{mole/flask}$). The results are shown in Table 3. Fructose oxidation was slightly decreased by low acetate concentrations and this decrease only became substantial at equi-molar concentrations of the two substrates. At the same time, these changes were reflected in an increase in the amount of acetate oxidized with increasing concentration. Statistical analyses of the results for both these parameters showed a significant quadratic, as well as a linear, component to the effect of increasing acetate content ($P < 0.01$). Although the addition of acetate had no significant effect on total oxygen uptake, it did significantly increase the oxygen uptake attributable to the combined added substrates ($P < 0.01$). The concomitant decrease in oxygen uptake not due to added substrate did not test as significant, nor was there a significant effect of acetate addition on the utilization of fructose or accumulation of lactate.

Four ejaculates of washed ram spermatozoa (3 to 6×10^8 cells/flask) were used to investigate the effects of partially replacing one substrate with an equal amount, on a carbon atom basis, of other substrate or substrates. The diluents contained 6 mM-fructose or 12 mM-sodium lactate or 18 mM-sodium acetate. Where combinations were studied, equal volumes of diluents were mixed so that all flasks during incubation contained equal numbers of substrate carbon atoms.

TABLE 3
METABOLISM OF WASHED RAM SPERMATOOZOA IN THE PRESENCE OF FRUCTOSE (15 μ MOLE/FLASK) AND
VARYING LEVELS OF SODIUM ACETATE

Molar ratio of fructose : acetate	O ₂ uptake	Fructose oxidized	Acetate oxidized	Fructose + acetate oxidized (O ₂ equivalent)	Other O ₂ uptake	Fructose utilized	Lactate accumulated
6 : 0	2.37	0.339	0.000	2.033	0.346	1.20	1.54
6 : 1/6	2.47	0.334	0.034	2.074	0.395	1.25	1.52
6 : 1	2.46	0.314	0.138	2.156	0.296	1.19	1.61
6 : 6	2.44	0.238	0.435	2.300	0.167	1.30	1.73
S.E. _m	0.070 (16)	0.010 (9)	0.016 (6)	0.051 (9)	0.076 (9)	0.037 (15*)	0.063 (16)

All values are expressed as μ mole/10⁸ spermatozoa over the experimental period (2 to 3 hr) and are the means for four ejaculates.

* d.f. reduced by 1 to correct for missing value.

The mean results are given in Tables 4 and 5. When fructose was partially replaced by lactate, the oxygen uptake was increased, both in the presence and absence of acetate. The amount of fructose oxidized was decreased by the inclusion of acetate or lactate, but the decrease was greater with lactate than with acetate. The amount of fructose utilized and the amount of lactate accumulated were not altered by the replacement of fructose by lactate or acetate. The replacement of lactate by fructose caused a fall in lactate oxidation (Table 5) and

TABLE 4

METABOLISM OF FRUCTOSE BY WASHED RAM SPERMATOZOA IN THE PRESENCE AND IN THE ABSENCE OF ALTERNATIVE SUBSTRATES

Substrate added ($\mu\text{mole}/\text{flask}$)	Total O_2 uptake	Fructose oxidized	Fructose utilized	Lactate accumulated
A. Fructose (15)	1.32	0.197	0.75	0.78
B. Fructose (7.5) + lactate (15)	1.44**	0.046**	0.80	0.63
C. Fructose (7.5) + acetate (22.5)	1.30	0.119*	0.64	0.76
D. Fructose (5) + lactate (10) + acetate (15)	1.45**	0.044**	0.72	0.52
S.E. _m	0.034 (16)	0.022 (9)	0.054 (9)	0.077 (16)

All values are expressed as $\mu\text{mole}/10^8$ spermatozoa over the experimental period (3 hr) and are the means for four ejaculates.

* Significantly different from A; $P < 0.05$.

** Significantly different from A; $P < 0.01$.

TABLE 5

OXIDATION OF LACTATE AND ACETATE BY WASHED RAM SPERMATOZOA IN THE PRESENCE AND IN THE ABSENCE OF OTHER SUBSTRATES

Substrate added ($\mu\text{mole}/\text{flask}$)	Lactate oxidized	Substrate added ($\mu\text{mole}/\text{flask}$)	Acetate oxidized
A. Lactate (30)	0.672	A. Acetate (45)	0.367
B. Lactate (15) + fructose (7.5)	0.543*	B. Acetate (22.5) + fructose (7.5)	0.282*
C. Lactate (15) + acetate (22.5)	0.660	C. Acetate (22.5) + lactate (15)	0.270*
D. Lactate (10) + fructose (5) + acetate (15)	0.441**	D. Acetate (15) + fructose (5) + lactate (10)	0.259*
S.E. _m	0.037 (9)		0.030 (9)

All values are expressed as $\mu\text{mole}/10^8$ spermatozoa over the experimental period (3 hr) and are the means for four ejaculates.

* Significantly different from A; $P < 0.05$.

** Significantly different from A; $P < 0.01$.

the replacement of acetate by fructose, lactate or both lactate and fructose caused a similar fall in acetate oxidation (Table 5).

The metabolism of washed ram spermatozoa (4 to 8×10^8 cells/flask) incubated for 3 hr in the presence of fructose ($15 \mu\text{mole}/\text{flask}$) and increasing levels of sodium citrate (1 , 3 and $9 \mu\text{mole}/\text{flask}$) were compared with incubations in fructose alone. The results for four ejaculates are given in Table 6. Addition of citrate gave a significant linear decrease in oxygen uptake and fructose oxidation ($P < 0.01$), but no change in the oxygen uptake attributable to substrates other than fructose. Although there was no effect on fructose

utilization, lactate accumulation was depressed by the addition of citrate, especially at the highest level. The intracellular level of isotopic carbon was measured in five ejaculates incubated for 3 hr in a diluent containing U- ^{14}C -fructose (15 $\mu\text{mole/flask}$; specific activity 6.67 $\text{m}\mu\text{C}/\mu\text{mole}$) with and without the addition of sodium citrate (9 $\mu\text{mole/flask}$). The mean concentration of isotopic carbon in the cell was found to increase significantly from 0.45 $\text{m}\mu\text{C}/10^8$ cells to 0.57 $\text{m}\mu\text{C}/10^8$ cells on the addition of citrate to the incubation medium ($t_{(4)} = 3.24$; $P < 0.05$).

TABLE 6

METABOLISM OF FRUCTOSE (15 $\mu\text{mole/flask}$) IN THE PRESENCE OF VARYING AMOUNTS OF SODIUM CITRATE

Citrate concentration ($\mu\text{mole/flask}$)	Total O_2 uptake	Fructose oxidized	Other O_2 uptake	Fructose utilized	Lactate accumulated
0	2.12	0.297	0.35	1.36	1.52
1	2.03	0.269	0.42	1.25	1.29
3	1.91	0.251	0.40	1.32	1.12
9	1.71	0.218	0.41	1.30	0.79
S.E. _m	0.074 (9)	0.011 (9)	0.036 (9)	0.175 (9)	0.067 (9)

All values are expressed as $\mu\text{mole}/10^8$ spermatozoa over the experimental period (3 hr) and are the means for four ejaculates.

TABLE 7

OXYGEN UPTAKE AND RECOVERY OF RADIOACTIVE CARBON DIOXIDE FOR RAM SPERMATOOZOA INCUBATED IN THE PRESENCE OF 1-5- ^{14}C -SODIUM CITRATE (5 $\mu\text{mole/flask}$) WITH AND WITHOUT THE ADDITION OF FRUCTOSE (5 $\mu\text{mole/flask}$)

Substrate added	O_2 uptake ($\mu\text{mole}/10^8$ cells)	$\text{m}\mu\text{C } ^{14}\text{CO}_2/\text{flask}$	% ^{14}C oxidized
Nil (control)	0.87	0.0	0.0
Citrate	0.87	1.453	2.90
Citrate + fructose	1.67**	1.486	2.96
S.E. _m	0.17 (8)	0.006 (4)	0.26 (4)

All values are the means for five ejaculates and are expressed as the total change over the experimental period (3 hr).

** Significantly different from control; $P < 0.01$.

In the next experiment, five ejaculates of washed ram spermatozoa (10 to 15×10^8 cells/flask) were incubated for 3 hr in the washing diluent, or in the presence of sodium citrate (5 $\mu\text{mole/flask}$), or in the presence of sodium citrate (5 $\mu\text{mole/flask}$) plus fructose (5 $\mu\text{mole/flask}$). To the flasks containing citrate, 100 $\text{m}\mu\text{C}$ of 1,5- ^{14}C -sodium citrate was added to gauge the oxidative utilization of this compound. The results are given in Table 7. The addition of citrate did not significantly alter the oxygen uptake when compared with the substrate-free incubation. Although fructose, in the presence of citrate, increased the oxygen uptake, its addition had no effect on the amount of $^{14}\text{CO}_2$ formed from the labelled citrate.

In the final experiment, four ejaculates of washed ram spermatozoa (7×10^8 cells/flask) were incubated for 3 hr in the presence of myo-inositol (1 μ mole/flask) and in the presence or absence of 20% (v/v) dialysed ram seminal plasma or 20% (v/v) dialysed fluid collected from the ewe's fallopian tube. In all flasks incubated, 0.4 m μ C of $^{14}\text{CO}_2$ was produced. The addition of ewe tubal fluid gave an increase in the oxygen uptake, but had no effect on the $^{14}\text{CO}_2$ produced.

DISCUSSION

It is now generally accepted that the in-vitro metabolism of spermatozoa in several mammalian species is predominantly of a glycolytic nature (Mann, 1964). Although the source of energy in washed spermatozoa incubated without added substrate is plasmalogen (Hartree & Mann, 1959, 1960a, b, 1961), the breakdown of phospholipid has not been shown to occur in semen in the presence of fructose. The respiratory quotient of 1 for ram semen obtained in the presence of fructose and lactate (Wales & O'Shea, 1966b) emphasizes the glycolytic nature of ram spermatozoal metabolism.

Fructose is the main free sugar of ram semen (Mann, 1964). Terner & Korsh (1962) have shown that spermatozoa are able to convert carbohydrate to lipids, but any nett synthesis of lipids from fructose must be of a minor nature, as fructose disappearing is nearly quantitatively accounted for as lactate and sorbitol accumulated and CO_2 formed (O'Shea & Wales, 1965). Sorbitol occurs in ram semen, but, in the presence of added fructose, does not materially contribute to the cell metabolism (O'Shea & Wales, 1965). Glycerylphosphorylcholine, although present in ram seminal plasma, only enters spermatozoal metabolism in the presence of enzymes of the female genital tract (White & Wallace, 1961). The occurrence of pyruvic acid in semen is in doubt (Mann, 1964).

In semen, glycolysis leads to a decrease in hexose concentration and an increase in the level of lactic acid (Mann, 1946; Scott, White & Annison, 1962a). As shown in the present paper, changes in the level of lactic acid in semen alter the amount of fructose oxidized to CO_2 . Thus, in the presence of physiological levels of lactate, the use of isotopically labelled fructose does not measure total substrate oxidation and lactate oxidation must also be considered. Changes in lactate concentration do not alter the rate of conversion of fructose to lactate unless the pH falls, in which case all indices of metabolism are decreased.

Fructose and lactic acid are probably oxidized to CO_2 by way of the Krebs' tricarboxylic acid cycle (Salisbury & Lodge, 1962). Scott, White & Annison (1962b) obtained suppression of acetate oxidation in ram spermatozoa by fluoroacetate, suggesting acetate also is oxidized by way of this cycle. As observed by Scott *et al.* (1962a), the addition of acetate decreased the oxidation of hexose. In the present studies, this decrease was less than with similar additions of lactate. This difference may be due to lactate forming a metabolic pool in the pathway of fructose utilization, while acetate acts as a competitor at the tricarboxylic level, or secondly it may be due to differences in availability

of the two compounds inside the cell. At comparable acetate levels to those occurring in ram semen (Scott, White & Annison, 1961), only a very small depression in fructose oxidation occurred and thus the oxidation of acetate in ram semen diluted with a fructose-containing diluent would not be a major source of energy.

The present results support the finding of Lardy & Phillips (1942) that citrate decreases the oxygen consumption of spermatozoa. The observation (I.C.A. Martin, unpublished data) that the addition of 10 mM-citrate depresses the motility and percentage motile in ram spermatozoa stored for 4 days at 5° C may be related to the depression in metabolism which occurs on the addition of citrate. At the level of citrate comparable to that present in semen diluted for metabolic experiments, however, the decrease in oxygen uptake and fructose oxidation is small and unimportant. As found by Humphrey & Mann (1949), fructose utilization was unaltered by citrate addition. However, high concentrations of added citrate depressed lactate accumulation. This depression in the conversion of fructose to lactate in the presence of citrate could be related to the reported inhibition of phosphofructokinase by citrate (Garland, Randle & Newsholme, 1963; Parmeggiani & Bowman, 1963; Passonneau & Lowry, 1963). The finding that the addition of citrate to the diluent for spermatozoa increased the concentration of intracellular labelled carbon in the presence of labelled fructose, indicates that a similar mechanism may operate in spermatozoa and lead to the accumulation of hexose phosphate in the cell.

In the experiment with labelled citrate, little citrate was oxidized and thus its oxidation neither compensates for any reduction in fructose oxidation nor materially adds to the oxygen uptake when fructose is present. As found by Humphrey & Mann (1949), the addition of sodium citrate to washed ram spermatozoa incubated without added substrate had no effect on oxygen uptake and the additional oxygen needed for citrate oxidation is probably too small to be measured by the methods used.

Inositol, which is present in the genital tract secretions of the female rabbit (Gregoire, Gongsakdi & Rakoff, 1962) and in seminal plasma (Mann, 1951, 1954; Hartree, 1957), is formed from glucose in the rat testis and seminal vesicles (Eisenberg, Bolden & Loewus, 1964; Imai, 1964). The fact that the $^{14}\text{CO}_2$ produced in the experiment with inositol was at the same low level in all flasks suggests that it may have been formed from a minor impurity in the ^{14}C -inositol. Thus no, or very little, CO_2 was formed from inositol in the presence of ram semen, with or without the addition of fluid obtained from the fallopian tube. Since the secretions of the fallopian tube did not affect the oxidation of inositol, it is unlikely that any enzymatic conversion of seminal inositol to a substrate for spermatozoa occurs in this part of the female genital tract.

From the foregoing experiments it may be concluded that, in the presence of added fructose, fructose and lactate are the main metabolites for unwashed ram spermatozoa under aerobic conditions. A quantitative measure of their oxidative metabolism can be obtained by using duplicate flasks for each treatment, one containing labelled fructose and the other labelled lactate. Assay

of radioactivity from these flasks coupled with an estimation of fructolysis should give a measure of the overall metabolism of ram spermatozoa.

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