# THE INFLUENCE OF THE FEMALE GENITAL TRACT ON THE METABOLISM OF RABBIT SPERMATOZOA

## II.* EFFECT OF STORAGE WITH GLUCOSE, LACTATE, BICARBONATE, AND FEMALE GENITAL TRACT FLUIDS

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*Summary*

In an attempt to isolate the factors responsible for altering the metabolism of spermatozoa incubated *in utero*, washed rabbit spermatozoa were stored *in vitro* for 6 hr at 37°C with either glucose, lactate, bicarbonate, or female genital tract fluids, and their ability to metabolize [1-$^{14}$C]glucose and [6-$^{14}$C]glucose was then compared with that of fresh spermatozoa.

Spermatozoa suffered a decline in oxygen uptake and glycolysis after storage for 6 hr and the yield of $^{14}CO_2$ from [6-$^{14}$C]glucose was less than that from [1-$^{14}$C]-glucose. Adding glucose or lactate to the medium lessened these senescence changes and the spermatozoa retained their ability to oxidize C-1 and C-6 of glucose at about the same rate. Low concentrations of uterine fluid, but not bicarbonate, had a similar effect. When higher concentrations of uterine fluid were included in the storage diluent with glucose, subsequent glycolytic activity, but not oxygen uptake, was depressed.

No addition to the incubation medium produced the changes characteristic of spermatozoa recovered from the uterus (i.e. increase in glycolysis, oxygen uptake, and oxidation of C-1 of glucose above the fresh controls).

It is concluded that these changes cannot be accounted for solely by the beneficial effects of genital tract fluids, glucose, or lactate, and some other unknown property of the uterus must also be involved. The evidence suggests that it is not bicarbonate.

## I. INTRODUCTION

Although over a decade has passed since the discovery that spermatozoa must spend 2–6 hr in the female reproductive tract before fertilization can occur (Austin 1951, 1952; Chang 1951; Noyes 1953), no biochemical changes in the spermatozoa have been unequivocally related to this process of capacitation. Recently, however, attempts have been made to approach the problem by comparing the metabolism of freshly ejaculated rabbit spermatozoa with that of spermatozoa after incubation in the uterus for 6 hr (Hamner and Williams 1963; Mounib and Chang 1964; Murdoch and White 1967). From this work, it appears that capacitated rabbit spermatozoa may have greater respiratory and glycolytic activity than freshly ejaculated spermatozoa, and may oxidize C-1 of the glucose molecule in preference to C-6, indicating

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a shift towards pentose shunt activity. Freshly ejaculated spermatozoa oxidize C-1 and C-6 of glucose at a similar rate suggesting that the sugar is metabolized predominantly via the Embden–Meyerhof glycolytic pathway.

Since both tubal and uterine fluid contain bicarbonate, which stimulates spermatozoal respiration and glycolysis (Hamner and Williams 1963, 1964; Murdoch and White 1968), it may in some way be related to the metabolic changes occurring in spermatozoa after incubation *in utero*. If, however, bicarbonate is involved, it must precondition the spermatozoa to such metabolic changes as they were washed free of luminal secretions after removal from the uterus and incubated in Warburg flasks containing potassium hydroxide to absorb carbon dioxide (Hamner and Williams 1963; Mounib and Chang 1964; Murdoch and White 1967).

In the present study an attempt has been made to isolate the factors involved in altering the metabolism of spermatozoa incubated *in utero*. This has been done by storing rabbit spermatozoa at 37°C for 6 hr *in vitro* with female genital tract fluids or their constituents (viz. glucose, bicarbonate, and lactate) and then comparing their metabolism with that of freshly collected spermatozoa.

## II. Materials and Methods

### (a) *Genital Tract Fluids*

Oviduct and uterine fluids were collected from oestrous does as described in Part I of this series (Murdoch and White 1968) and used within 30 min.

### (b) *Radioactive Glucose and Unlabelled Substrates*

D-[1-$^{14}$C]- and D-[6-$^{14}$C]glucose, obtained from the Radiochemical Centre, Amersham, England, and unlabelled glucose and lactate were added to calcium-free Krebs–Ringer phosphate buffer (Umbreit, Burris, and Stauffer 1959) to give the desired concentration and radioactivity. The diluent contained streptomycin and penicillin (0·5 mg/ml of each). The radioactivity of the labelled substrates was checked by counting in a Nuclear Chicago liquid scintillation spectrophotometer.

### (c) *Preparation and Incubation of Spermatozoal Suspensions*

Rabbit semen was collected with an artificial vagina (White 1955). Only samples of good initial motility were used. Ejaculates were pooled and divided into two portions designated "fresh" and "stored". The spermatozoa were washed in sterile calcium-free Krebs–Ringer phosphate buffer (pH 7·4) by the procedure previously described in Part I. Metabolic studies were undertaken on the fresh samples within 30 min of collection and on the stored samples after keeping in Warburg flasks. All flasks were shaken at 114 strokes/min at 37°C.

(i) *Fresh Spermatozoa.*—Washed spermatozoal suspensions (0·5 ml, $1{\cdot}5–3{\cdot}6 \times 10^8$ cells per flask) were incubated with either D-[1-$^{14}$C]- or D-[6-$^{14}$C]glucose (0·5 ml, 9 $\mu$moles) in Warburg flasks of 6 ml capacity for 2 hr. Oxygen uptake was measured with air as gas phase and 0·05 ml 20% (w/v) KOH in the centre well.

(ii) *Stored Spermatozoa.*—Spermatozoa ($3{\cdot}4–7{\cdot}1 \times 10^8$ cells per flask) were stored in a final volume of 3 ml in Warburg flasks of 15–18 ml capacity for 6 hr. Samples containing bicarbonate or genital tract fluids were incubated in side-arm flasks and under a 2% $CO_2$ atmosphere (see Part I). Otherwise, flasks without side-arms were used with 0·2 ml 20% (w/v) KOH in the centre well. Precise experimental details are given in Section III. At the end of the storage period the sperm suspension was diluted threefold with sterile washing diluent and centrifuged at 400 *g* for 10 min. The supernatant was discarded and the spermatozoa resuspended in a convenient volume of diluent for metabolic studies as undertaken with the fresh samples.

### (*d*) *Analytical Methods*

Glucose and lactate were estimated in protein-free extracts of the spermatozoal suspension at the beginning and end of all metabolic studies by procedures previously described (Murdoch and White 1968). The $^{14}CO_2$ produced by the spermatozoa from D-[1-$^{14}$C]- and D-[6-$^{14}$C]glucose was assayed as $Ba^{14}CO_3$ by the procedure of Annison and White (1961).

### (*e*) *Statistical Analyses*

The results of all experiments were subjected to standard analysis of variance. In experiment 2 the residual mean squares are given in italics at the base of the variance ratio columns in the analysis of variance (Table 2). In all other experiments, the residual mean square has been used to calculate the standard errors of the difference between means and this has been used in *t*-tests.

## III. Experimental Procedure and Results

### (*a*) *Experiment 1: Metabolism of Labelled Glucose after Storage in the Presence or Absence of Oviduct and Uterine Fluids*

Spermatozoal suspensions (2·2 ml) were stored in Warburg flasks with 0·8 ml of 0·9% (w/v) NaCl or oviduct or uterine fluid containing glucose (18 μmoles).

TABLE 1

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY RABBIT SPERMATOZOA AFTER STORAGE FOR 6 HR IN THE PRESENCE OF GLUCOSE WITH AND WITHOUT OVIDUCT AND UTERINE FLUIDS

Values are calculated per $10^8$ spermatozoa per 2 hr and represent the means of four ejaculates. 500 nCi of glucose were added to each flask

| Treatment | Oxygen Uptake (μl) | Glucose Utilized (μmoles) | Lactate Produced (μmoles) | Yield of $^{14}CO_2$ (nCi) from: [6-$^{14}$C]Glucose (A) | Yield of $^{14}CO_2$ (nCi) from: [1-$^{14}$C]Glucose (B) | A/B |
|---|---|---|---|---|---|---|
| Fresh sample | 15·5 | 1·86 | 2·71 | 4·57 | 5·21 | 0·88 |
| Sample stored with: | | | | | | |
| Glucose | 7·7** | 1·52 | 2·21 | 1·52** | 1·96** | 0·62* |
| Glucose + oviduct fluid | 10·8* | 1·66 | 2·43 | 2·70* | 3·15* | 0·86 |
| Glucose + uterine fluid | 13·5 | 1·91 | 2·78 | 3·78 | 4·04 | 0·94 |
| Mean standard errors | 2·05 | 0·40 | 0·62 | 0·75 | 0·81 | 0·10 |

* Significantly different from fresh sample, $P < 0·05$.

** Significantly different from fresh sample, $P < 0·01$.

Table 1 shows that, although in no case after storage was the oxygen uptake, glycolysis, or oxidation of C-1 of glucose greater than that of fresh spermatozoa, the metabolic activity of spermatozoa stored with uterine fluid was as good as that of the fresh samples. Glycolytic activity did not deteriorate significantly under any condition of storage although the respiratory activity of spermatozoa stored in oviduct fluid or glucose alone was depressed, particularly in the latter instance. The ratio of yields of $^{14}CO_2$ from [6-$^{14}$C]- and [1-$^{14}$C]glucose was slightly lower than for fresh samples after storing in glucose alone whereas the C-6 : C-1 oxidation ratio of samples stored with genital tract fluids was about the same as for the fresh samples.

### (*b*) *Experiment 2: Metabolism of Labelled Glucose after Storage in the Presence or Absence of Uterine Fluid and Glucose*

This experiment was designed to discover whether storage of spermatozoa with larger amounts of fluid in the presence or absence of glucose might enhance respiratory and glycolytic activity compared with that of fresh samples.

Spermatozoal suspensions (1 ml) were stored in Warburg flasks with glucose (18 $\mu$moles) or 2 ml of uterine fluid or a combination of both. Control flasks contained 2 ml of 0·9% (w/v) NaCl. The results and a summary of the analysis of variance are given in Table 2.

TABLE 2

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY RABBIT SPERMATOZOA AFTER STORAGE FOR 6 HR IN THE PRESENCE AND ABSENCE OF UTERINE FLUID AND GLUCOSE

Values are calculated per $10^8$ spermatozoa per 2 hr and represent the means of three ejaculates. 100 nCi of glucose were added to each flask

| Treatment | Oxygen Uptake ($\mu$l) | Glucose Utilized ($\mu$moles) | Lactate Produced ($\mu$moles) | Yield of $^{14}CO_2$ (nCi) from: [6-$^{14}$C]Glucose (A) | Yield of $^{14}CO_2$ (nCi) from: [1-$^{14}$C]Glucose (B) | A/B |
|---|---|---|---|---|---|---|
| Fresh sample | 17·5 | 1·71 | 2·43 | 1·01 | 1·05 | 0·97 |
| Sample stored with: | | | | | | |
| Saline | 1·7 | 0·02 | 0·03 | 0·002 | 0·010 | 0·24 |
| Glucose | 8·9 | 1·27 | 1·79 | 0·13 | 0·26 | 0·46 |
| Uterine fluid | 9·7 | 1·32 | 1·52 | 0·17 | 0·28 | 0·59 |
| Uterine fluid + glucose | 10·0 | 0·49 | 0·51 | 0·25 | 0·33 | 0·75 |

Summary of Analysis of Variance

| Source of Variation | D.F. | Variance Ratios: Oxygen Uptake | Glucose Utilized | Lactate Produced | $^{14}CO_2$ from C-6 of Glucose (A) | $^{14}CO_2$ from C-1 of Glucose (B) | A/B |
|---|---|---|---|---|---|---|---|
| Treatments | (4) | | | | | | |
| Fresh *v.* stored | 1 | 46·7** | 23·4** | 38·7** | 70·0** | 65·6** | 23·7** |
| Effect of fluid | 1 | 12·4** | 2·2 | 0·3 | 2·4 | 3·4 | 14·3** |
| Effect of glucose | 1 | 8·3* | 1·4 | 3·1 | 1·3 | 2·6 | 5·3* |
| Interaction: | | | | | | | |
| Fluid × glucose | 1 | 7·1* | 35·9** | 42·8** | 0·04 | 1·3 | 0·1 |
| Ejaculates | 2 | 5·7* | 2·9 | 3·4 | 3·4 | 5·8* | 1·0 |
| Residual | 8 | *5·05* | *0·09* | *0·13* | *0·03* | *0·03* | *0·02* |

* $P < 0·05$. ** $P < 0·01$.

None of the storage conditions raised respiratory or glycolytic activity or the oxidation of the C-1 of glucose above that of fresh spermatozoa and samples stored only in saline had very little metabolic activity and low C-6 : C-1 oxidation ratios. The presence of glucose or uterine fluid during storage clearly increased the subsequent metabolic activity of the spermatozoa and increased the ratio of $^{14}CO_2$ yields from [6-$^{14}$C]- and [1-$^{14}$C]glucose. The glycolytic activity of spermatozoa stored with both glucose and uterine fluid was much less than that of spermatozoa stored with either factor alone although this was not true for respiratory activity.

### (c) *Experiment 3: Metabolism of Labelled Glucose after Storage in the Presence or Absence of Bicarbonate*

Hamner and Williams (1964) and Murdoch and White (1968) have shown that bicarbonate is the factor in tubal and uterine fluids stimulating spermatozoal metabolism. It was of interest, therefore, to study the metabolism of spermatozoa after storage in the presence of bicarbonate to see if it would produce a response similar to that obtained with oviduct and uterine fluids in experiment 1.

Spermatozoa were stored in Warburg flasks in 3 ml of sterile diluent containing 18 μmoles of glucose and 6·00 m-moles/l of sodium bicarbonate. Table 3 shows that the respiratory activity of stored spermatozoa, incubated in the presence or absence of bicarbonate, was significantly lower than that of fresh spermatozoa. Glycolysis, however, proceeded at the same rate in stored and fresh samples. In stored spermatozoa, the oxidation of the C-1 of glucose declined to a lesser extent than that of C-6, again giving low C-6 : C-1 oxidation ratios.

TABLE 3

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY RABBIT SPERMATOZOA AFTER STORAGE FOR 6 HR IN THE PRESENCE OF GLUCOSE WITH AND WITHOUT BICARBONATE

Values are calculated per $10^8$ spermatozoa per 2 hr and represent the means of four ejaculates. 500 nCi of glucose were added to each flask

| Treatment | Oxygen Uptake (μl) | Glucose Utilized (μmoles) | Lactate Produced (μmoles) | Yield of $^{14}CO_2$ (nCi) from: [6-$^{14}$C]Glucose (A) | Yield of $^{14}CO_2$ (nCi) from: [1-$^{14}$C]Glucose (B) | A/B |
|---|---|---|---|---|---|---|
| Fresh sample | 20·4 | 2·14 | 2·89 | 6·46 | 6·47 | 1·00 |
| Sample stored with: | | | | | | |
| Glucose | 7·7** | 2·14 | 3·01 | 1·04** | 1·45** | 0·60* |
| Glucose + bicarbonate | 8·1** | 2·19 | 2·95 | 1·02** | 1·64** | 0·56* |
| Mean standard errors | 0·64 | 0·24 | 0·36 | 0·41 | 0·24 | 0·13 |

* Significantly different from fresh sample, $P < 0·05$.

** Significantly different from fresh sample, $P < 0·01$.

### (d) *Experiment 4: Metabolism of Labelled Glucose after Storage in the Presence of Bicarbonate, Lactate, and Protein*

The respiration of rabbit spermatozoa is particularly high in the presence of bicarbonate when the substrate is lactate, which also promotes the maintenance of motility (Murdoch and White 1968). The inclusion of protein in diluents is known to protect spermatozoa to some extent from the harmful effects of dilution (Emmens and Swyer 1948). Spermatozoa were stored in Warburg flasks in 3 ml of sterile diluent containing bicarbonate (6·00 mM), lactate (14 μmoles) and 4 mg of bovine serum albumin.

The respiratory and glycolytic activity of stored spermatozoa was significantly lower than fresh samples, and in the absence of substrate stored spermatozoa had very little metabolic activity and low C-6 : C-1 ratios (Table 4). Spermatozoa stored in the presence of lactate yielded similar amounts of $^{14}CO_2$ from [6-$^{14}$C]glucose and [1-$^{14}$C]glucose but oxidized the sugars at a slower rate than fresh cells. Adding protein before storage had no effect on the subsequent metabolism of the spermatozoa.

TABLE 4

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY RABBIT SPERMATOZOA AFTER STORAGE FOR 6 HR WITH BICARBONATE, LACTATE, AND PROTEIN

Values are calculated per $10^8$ spermatozoa per 2 hr and represent the means of three ejaculates. 100 nCi of glucose were added to each flask

| Treatment | Oxygen Uptake ($\mu$l) | Glucose Utilized ($\mu$moles) | Lactate Produced ($\mu$moles) | Yield of $^{14}CO_2$ (nCi) from: [6-$^{14}$C]Glucose (A) | Yield of $^{14}CO_2$ (nCi) from: [1-$^{14}$C]Glucose (B) | A/B |
|---|---|---|---|---|---|---|
| Fresh sample | 15·4 | 1·49 | 2·03 | 0·94 | 1·06 | 0·89 |
| Sample stored with: | | | | | | |
| Bicarbonate | 0·9** | 0·05** | 0·04** | 0·004** | 0·012** | 0·35** |
| Bicarbonate + lactate | 11·3* | 1·19** | 1·53** | 0·57* | 0·69* | 0·81 |
| Bicarbonate + lactate + protein | 11·0* | 1·21* | 1·58** | 0·61* | 0·66* | 0·92 |
| Mean standard errors | 1·46 | 0·08 | 0·07 | 0·10 | 0·13 | 0·09 |

* Significantly different from fresh sample, $P < 0·05$.

** Significantly different from fresh sample, $P < 0·01$.

## IV. DISCUSSION

Spermatozoa stored without an exogenous substrate clearly suffer a marked decline in aerobic glycolysis and respiration in the presence of added glucose, when compared with fresh samples. The yield of $^{14}CO_2$ from [6-$^{14}$C]glucose declined more than that from [1-$^{14}$C]glucose when the spermatozoa were stored with glucose alone, or in the absence of an added substrate, and supports the suggestion of Murdoch and White (1967) that the pentose phosphate pathway may be more resistant to senescence changes than the tricarboxylic acid cycle. It is evident from the present study that the inclusion of glucose in the medium for storing rabbit spermatozoa at 37°C prevents deterioration in subsequent glycolytic activity and, to some extent, oxygen uptake. Adding uterine fluid has a similar stabilizing effect on subsequent oxygen uptake and may even enhance the action of glucose and the ability of stored spermatozoa to oxidize C-1 and C-6 of glucose at about the same rate. The effect on glucose metabolism does not appear to be due to the bicarbonate content of uterine fluid. The lactate content of uterine fluid may, however, be responsible to some extent for the maintenance of oxidative capacity as spermatozoa stored in the presence of this substrate displayed only a slight, though significant, decline in their ability to oxidize C-1 and C-6 of glucose, and oxidized both carbon atoms at approximately the same rate.

When added to sperm suspensions prior to storage, uterine fluid by itself maintained subsequent glycolytic activity at a high rate. The detrimental effect of the larger volume of uterine fluid in the presence of glucose might be due to excessive production of lactic acid during storage. Murdoch and White (1968) have shown that the rate of breakdown of glucose to lactic acid by rabbit spermatozoa is considerably enhanced in the presence of uterine fluid.

After 6 hr in the uterus of the oestrous rabbit the oxygen uptake, glycolytic activity, and ability of rabbit spermatozoa to oxidize C-1 of the glucose molecule are increased above that of fresh samples (Hamner and Williams 1963; Mounib and Chang 1964; Murdoch and White 1967). Failure in the present study to achieve this after 6 hr storage *in vitro* indicates that the phenomenon is not merely due to the beneficial effects of genital tract fluids or to bicarbonate (Hamner and Williams 1964; Murdoch and White 1968), and that some other unknown property of the uterus must also be involved.

The possibility of the uterus selectively expelling poorer spermatozoa and retaining better ones is excluded since the uterus was ligated in the studies of Murdoch and White (1967). Preferential elimination of poorer spermatozoa by passage into the genital tract epithelium (Posalaky and Törő 1957; Austin and Bishop 1959), or by phagocytosis (Austin 1957; Howe and Black 1963; Bedford 1965; Reid 1965) is unlikely in such studies since spermatozoa have apparently never been seen in the uterine glands of the rabbit (see Sobrero 1963) and the spermatozoa were generally removed from the uterus before major leucocyte infiltration occurred. Bedford (1965) has shown that only spermatozoa with damaged acrosomes or damaged head membranes are ingested to any extent in the pseudopregnant uterus or following *in vitro* incubation with leucocytes obtained from the oestrous uterus. However, even if some selective elimination of poorer spermatozoa occurs in the uterus it is doubtful if this could account for the alteration in the oxidation of C-1 and C-6 of the glucose molecule by spermatozoa incubated *in utero*, and it seems most likely to arise from a true change in the cell itself. It has yet to be established if such changes in spermatozoal metabolism are an essential feature of capacitation.

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## VI. References

Annison, E. F., and White, R. R. (1961).—*Biochem. J.* **80**, 162.
Austin, C. R. (1951).—*Aust. J. scient. Res.* B **4**, 581.
Austin, C. R. (1952).—*Nature, Lond.* **170**, 326.
Austin, C. R. (1957).—*J. Endocr.* **14**, 335.
Austin, C. R., and Bishop, M. W. H. (1959).—*J. Endocr.* **18**, viii.
Bedford, J. M. (1965).—*J. Reprod. Fert.* **9**, 249.
Chang, M. C. (1951).—*Nature, Lond.* **168**, 697.
Emmens, C. W., and Swyer, G. I. M. (1948).—*J. gen. Physiol.* **32**, 121.

HAMNER, C. E., and WILLIAMS, W. L. (1963).—*J. Reprod. Fert.* **5**, 143.
HAMNER, C. E., and WILLIAMS, W. L. (1964).—*Proc. Soc. exp. Biol. Med.* **117**, 240.
HOWE, G. R., and BLACK, D. L. (1963).—*J. Reprod. Fert.* **6**, 305.
MOUNIB, M. S., and CHANG, M. C. (1964).—*Nature, Lond.* **201**, 943.
MURDOCH, R. N., and WHITE, I. G. (1967).—*J. Reprod. Fert.* **14**, 213.
MURDOCH, R. N., and WHITE, I. G. (1968).—*Aust. J. biol. Sci.* **21**, 961–72.
NOYES, R. W. (1953).—*West. J. Surg. Obstet. Gynec.* **61**, 342.
POSALAKY, Z., and TÖRŐ, I. (1957).—*Nature, Lond.* **179**, 150.
REID, B. L. (1965).—*Aust. J. Zool.* **13**, 189.
SOBRERO, A. J. (1963).—In "Mechanisms Concerned with Conception". p. 173. (Ed. C. G. Hartman.) (Pergamon Press: New York.)
UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. (1959).—"Manometric Techniques in Tissue Metabolism." (Burgess Publ. Co.: Minneapolis.)
WHITE, I. G. (1955).—*Aust. J. exp. Biol.* **33**, 367.