THE FALLOPIAN TUBE OF THE SHEEP

IV.* THE METABOLISM OF RAM SPERMATOZOA IN THE PRESENCE OF FLUID FROM THE FALLOPIAN TUBE

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Summary

Using isotopically labelled substrates, the metabolism of ram spermatozoa was examined in the presence of fluid from the fallopian tubes of normal ewes and spayed ewes receiving oestrogen and progesterone in factorial combination. The oxygen uptake of spermatozoa in tubal fluid was variable but was generally less than that of spermatozoa incubated in a saline diluent containing glucose (control). Due to the presence of lactate in the tubal fluids, the oxidation of added glucose by spermatozoa was consistently depressed when compared with the saline controls. On the other hand glucose utilization and lactate accumulation by spermatozoa were stimulated in the presence of tubal fluids in all experiments, the response being generally twice that of the controls. In addition, similar effects were found in fluids collected during two consecutive oestrous cycles.

The hormonal treatments had little effect on the metabolism of spermatozoa. There were no differences in response to fluids collected during different stages of the oestrous cycle.

I. INTRODUCTION

There are few reports concerning the effects of the fluids from the female genital tract on the metabolism of spermatozoa. Recently Hamner and Williams (1963) and Mounib and Chang (1964) have shown that capacitated rabbit spermatozoa respire at a greater rate than freshly ejaculated spermatozoa. Olds and Van Demark (1957) and Hamner and Williams (1963) have found that tubal fluid stimulates the respiration of spermatozoa. However, such changes are yet to be related directly to any physiological function, such as capacitation.

Since spermatozoa show marked changes in metabolism in response to alteration of their chemical environment (see Mann 1954) it is important to determine the changes brought about by the fluids of the female tract. Work of this nature could provide clues as to the physiological significance of the environment in the female tract and for this reason the metabolism of spermatozoa incubated in tubal fluids was studied.

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II. Materials and Methods

(a) *General*

Twenty ewes with cannulae in their fallopian tubes were treated as follows during each cycle period of 17 days:

(1) 4 spayed ewes were given 10 mg/day intramuscularly of progesterone on days 2–13 inclusive, followed by 30 μg intramuscularly of oestradiol benzoate on day 15.

(2) 4 spayed ewes were given 30μg intramuscularly of oestradiol benzoate on day 15.

(3) 4 spayed ewes were given 10 mg/day intramuscularly of progesterone on days 2–13 inclusive.

(4) 4 spayed ewes were given no treatment.

(5) 4 normal cycling ewes received no treatment; day 1 of the cycle of these ewes was taken as the day on which oestrus was first detected.

The ewes received one cycle of treatment before observations commenced on two subsequent cycles.

The 17-day treatment cycle was arbitrarily divided into three periods henceforth referred to as stage 1 (days 1 and 2), stage 2 (days 3–7), and stage 3 (days 8–16). In the normal cycling ewes in which the oestrous cycle exceeded 17 days, stage 3 was extended. In the spayed ewes receiving oestradiol benzoate and in the normal ewes, the three stages correspond approximately to the physiological states of oestrus (stage 1), metoestrus (stage 2), and dioestrus (stage 3). In the other two groups of ewes, in which a cyclic pattern was not expected, the division into stages is purely for comparison. This experimental design has been discussed in detail by Restall (1966).

Tubal fluid from each ewe was collected daily and the collections were grouped into the three stages defined above. The pooled fluids were frozen at -30°C until the experiments could be performed.

(b) *Semen*

Ejaculated semen was collected by electrical stimulation of the ram with a bipolar rectal probe as described by Blackshaw (1954). Only samples of good initial motility were used, and care was taken to avoid sudden temperature changes during collection and handling.

After collection, the spermatozoa were washed twice in a diluent composed of 40 mM mono- and disodium phosphate buffer (pH 7·0) and 100 mM sodium chloride. One volume of semen was diluted to 10 volumes and centrifuged at 200 g for 7 min. The supernatant was then removed, the spermatozoa resuspended, and again centrifuged. After removing the second supernatant the spermatozoa were resuspended in the diluent to give an approximate concentration of $3–6 \times 10^8$ cells per millilitre.

(c) *Incubation of Spermatozoa*

Warburg flasks of 5 ml capacity contained 0·3 ml of washed spermatozoal suspension ($1–2 \times 10^8$ cells per flask), 0·3 ml of tubal fluid, and 0·2 ml of substrate diluent [40 mM phosphate buffer (pH 7·0), 75 mM sodium chloride, 50 mM glucose]

and 0·05 ml of 20% (w/v) KOH in the centre well. Pairs of Warburg flasks were prepared, 0·1 ml of [U-^{14}C]glucose being added to one and to the other 0·1 ml of sodium [1-^{14}C]lactate as carrier-free isotope in 0·9% sodium chloride. By measurement of the initial specific activities of the glucose and lactate, the oxidation of these

TABLE 1

DESIGN OF THE EXPERIMENT SHOWING CLASSIFICATIONS INTO WHICH EACH FLUID WAS ASSIGNED

Each block of two ejaculates (e.g. ejac. 1 + ejac. 2) represents one Warburg run. G, Warburg flask containing [U-^{14}C]glucose. L, Warburg flask containing sodium [1-^{14}C]lactate

Treatment	Stage of Cycle	Cycle 1 or 2	
		Ewe 1	Ewe 2
Normal ewes	Control	*Ejac. 1* + *Ejac. 2*: G G / G G	*Ejac. 3* + *Ejac. 4*: G G / G G
	Stage 1	G L / G L	G L / G L
	Stage 2	G L / G L	G L / G L
	Stage 3	G L / G L	G L / G L
Spayed ewes, progesterone + oestradiol benzoate	Control	*Ejac. 5* + *Ejac. 6*: G G / G G	*Ejac. 7* + *Ejac. 8*: G G / G G
	Stage 1	G L / G L	G L / G L
	Stage 2	G L / G L	G L / G L
	Stage 3	G L / G L	G L / G L
Spayed ewes, oestradiol benzoate alone	Control	*Ejac. 9* + *Ejac. 10*: G G / G G	*Ejac. 11* + *Ejac. 12*: G G / G G
	Stage 1	G L / G L	G L / G L
	Stage 2	G L / G L	G L / G L
	Stage 3	G L / G L	G L / G L
Spayed ewes, progesterone alone	Control	*Ejac. 13* + *Ejac. 14*: G G / G G	*Ejac. 15* + *Ejac. 16*: G G / G G
	Stage 1	G L / G L	G L / G L
	Stage 2	G L / G L	G L / G L
	Stage 3	G L / G L	G L / G L
Spayed ewes, no hormone treatment	Control	*Ejac. 17* + *Ejac. 18*: G G / G G	*Ejac. 19* + *Ejac. 20*: G G / G G
	Stage 1	G L / G L	G L / G L
	Stage 2	G L / G L	G L / G L
	Stage 3	G L / G L	G L / G L

substrates was calculated after assay of the trapped carbon dioxide from the centre well of the flasks. Duplicate control flasks contained 0·3 ml of 0·9% sodium chloride instead of tubal fluid, and 0·1 ml of [U-^{14}C]glucose.

The flasks were incubated in the Warburg apparatus at 37°C for 3 hr and oxygen uptake was measured directly (Umbreit, Burris, and Stauffer 1959). The oxygen uptake not accounted for by the oxidation of glucose and lactate has been termed "other oxygen uptake".

(*d*) *Analytical Methods*

After incubation, protein-free extracts were prepared by precipitation with equal volumes of 0·3N barium hydroxide and 5% (w/v) zinc sulphate. Glucose and lactate were estimated by enzymic methods (Barker and Britton 1957; Huggett and Nixon 1957). The $^{14}CO_2$ produced by the spermatozoa from ^{14}C-labelled substrates was assayed as barium carbonate by the procedure of Annison and White (1961). After determining the initial and final levels of lactate and glucose in the flasks, the total amount of glucose utilized, the amount of lactate accumulated, and the amount of lactate formed could be calculated. The term "lactate accumulated" refers to the difference between final and initial lactate levels and the term "lactate formed" refers to the lactate accumulated plus that oxidized.

(*e*) *Experimental Design and Analysis*

The small quantities of fluid available and the fact that the Warburg apparatus held only 16 reaction flasks placed restrictions on the design of the experiment and subsequent analyses. Each Warburg experiment consisted of measuring the metabolism of the spermatozoa from two ejaculates in the fluid from three stages of the cycle from one ewe and in a saline control. Thus with duplicate flasks to obtain the isotope data, 16 reaction flasks were used for each ewe. Tubal fluids from two ewes in any treatment group were used and, by necessity, different ejaculate pairs were used in the fluids from each ewe. In one treatment cycle, therefore, fluids from 10 ewes were examined and 20 ejaculates were used. The design of the experiment is set out in Table 1.

The data for each parameter were subjected to analyses of variance. With the exception of parameters obtained from isotopic data, duplicate readings were obtained for all parameters measured. As the two ewes used as sources of fluid in any treatment group for the first cycle of treatment were not necessarily the two ewes used as sources of fluid in the second cycle of treatment, the data from the two cycles were analysed separately. The analyses of variance were complicated by the design of the experiment and because of the interdependence of some treatments the significance tests were carried out as shown in Table 2 (see Cochrane and Cox 1957). Some of the computations were carried out by a digital computer (SILLIAC, University of Sydney), using the programs of Dr. P. J. Claringbold.

III. Results

Summaries of the results are given in Table 3 (first cycle of treatment) and Table 4 (second cycle of treatment) with the respective analyses of variance shown in Tables 5 and 6. In fluids from normal ewes in both cycles of treatment, oxygen uptake and glucose oxidation by the spermatozoa was generally less than in the saline controls. On the other hand, total glucose utilized, lactate accumulated, and lactate formed were greater in the fluids than in the controls. The magnitude of this response varied between ejaculates and between ewes but was generally twice that of the saline controls (Table 7). Fluids from the normal ewes produced the same effects irrespective of whether they were from the first or second oestrous cycle.

In addition there were no differences between fluids from different stages of the oestrous cycle but there was considerable variation between ewes and between ejaculates, especially in regard to respiration.

The metabolism of spermatozoa incubated in fluids collected from spayed ewes was similar to that seen in normal ewes (Table 7). The respiration of the spermatozoa was not affected by the treatments imposed on the spayed ewes in the first cycle of treatment, but in those ewes receiving progesterone, glucose utilization and lactate

TABLE 2

PLAN OF THE ANALYSES OF VARIANCE PERFORMED ON THE EXPERIMENTAL DATA

This shows the source of variation, the degrees of freedom, the derivation of each variance, and the mean squares used for the test of significance

Source of Variation	Reference Notation	Degrees of Freedom	Derivation of Variance Component	Mean Square Used for Testing of Significance
Between ewes (A)	(a)	9	Computed	
Between treatments	(b)	(4)	Computed	←
Between ewes within treatment	(c)	(5)	(a) − (b)	←
Between stages of cycle (B)	(d)	3	Computed	* ←
$A \times B$ interactions	(e)	27	Stage × ewes subclass [(a) − (d)]	
Stage of cycle × treatment	(f)	(12)	Computed	←
Stage of cycle × ewes within treatment	(g)	(15)	(e) − (f)	←
Between ejaculates within ewes	(h)	10	Ejaculates sum of squares − (a)	←
Ejaculates within ewes × stages	(i)	30	Residual	←
Duplicates	(j)	80	Computed from duplicate	←

*If (g) not significant, then tests made with mean square for (i).

accumulation was greatly decreased (Table 3). However, in the second cycle, fluids derived from those spayed ewes receiving progesterone lowered oxygen uptake and glucose oxidation but did not affect glycolysis significantly when compared with ewes not receiving progesterone. There were no differences between fluids derived from different stages of the cycle.

The oxidation of lactate by the spermatozoa showed a significant variation between ewes within the hormone treatments, but there was no similar effect in any of the other parameters examined.

In general, ejaculate and ewe interactions accounted for a large proportion of the observed variation particularly where effects of the stage of cycle were concerned. This large variation between ejaculates or ewes was observed in most parameters measured.

TABLE 3

METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUIDS FROM NORMAL AND SPAYED EWES RECEIVING HORMONE TREATMENTS

All values are expressed as μmoles/10^8 cells over the experimental period (3 hr). The data are means for two ejaculates in two fluid samples from the first treatment cycle following cannulation of the fallopian tube. P = progesterone; ODB = oestradiol benzoate; C = saline control

Treatment	Stage of cycle	Total Oxygen Uptake	Oxygen Uptake due to Glucose Oxidation	Oxygen Uptake due to Lactate Oxidation	Other Oxygen Uptake	Glucose Utilized	Lactate Accumulated	Lactate Formed
Normal ewes	C	2·17	1·86	—	0·34	1·06	1·43	1·43
	1	2·31	1·32	0·193	0·73	2·85	4·51	4·64
	2	1·94	1·02	0·177	0·70	2·49	4·15	4·25
	3	1·32	0·69	0·166	0·42	2·09	3·62	3·73
Mean*		1·86	1·01	0·179	0·62	2·48	4·09	4·21
Spayed ewes, P+ODB	C	2·03	1·78	—	0·25	1·12	1·32	1·32
	1	1·48	0·70	0·294	0·49	1·71	2·22	2·42
	2	1·34	0·74	0·187	0·41	1·95	3·10	3·23
	3	1·29	0·78	0·263	0·25	2·05	2·84	3·02
Mean*		1·37	0·74	0·248	0·38	1·90	2·72	2·89
Spayed ewes, ODB alone	C	2·15	1·62	—	0·53	1·08	1·41	1·41
	1	1·64	1·07	0·054	0·52	2·56	4·53	4·58
	2	1·52	0·94	0·028	0·55	2·56	4·37	4·39
	3	1·46	0·87	0·027	0·57	2·73	4·88	4·91
Mean*		1·54	0·96	0·036	0·55	2·62	4·59	4·63
Spayed ewes, P alone	C	2·32	2·00	—	0·32	1·00	1·45	1·45
	1	0·94	0·55	0·145	0·26	1·91	3·20	3·34
	2	0·76	0·48	0·154	0·13	1·44	3·03	3·18
	3	1·09	0·74	0·082	0·27	1·69	3·71	3·79
Mean*		0·93	0·59	0·127	0·22	1·68	3·33	3·44
Spayed ewes, no treatment	C	2·86	2·37	—	0·53	1·44	2·27	2·27
	1	1·16	0·75	0·236	0·18	2·55	4·41	4·64
	2	1·55	1·04	0·206	0·30	2·99	5·22	5·44
	3	1·30	0·84	0·193	0·28	2·94	4·99	5·15
Mean*		1·34	0·88	0·211	0·26	2·83	4·87	5·08

*Does not include saline control.

TABLE 4

METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUID FROM NORMAL EWES AND SPAYED EWES RECEIVING HORMONE TREATMENTS

All values are expressed in μmoles/10^8 cells over the experimental period (3 hr). The data are means for two ejaculates in each of two fluid samples from ewes in the second treatment cycle following cannulation of the fallopian tube. P = progesterone; ODB = oestradiol benzoate; C = saline control

Treatment	Stage of Cycle	Total Oxygen Uptake	Oxygen Uptake due to Glucose Oxidation	Oxygen Uptake due to Lactate Oxidation	Other Oxygen Uptake	Glucose Utilized	Lactate Accumulated	Lactate Formed
Normal ewes	C	2·48	2·02	—	0·45	1·40	2·01	2·01
	1	2·21	1·39	0·158	0·67	3·19	5·45	5·53
	2	2·46	1·54	0·143	0·78	3·12	5·23	5·31
	3	2·43	1·32	0·338	0·77	3·12	5·25	5·41
Mean*		2·37	1·42	0·213	0·74	3·15	5·31	5·42
Spayed ewes, P+ODB	C	1·56	1·20	—	0·36	1·16	1·13	1·13
	1	1·31	0·65	0·168	0·49	2·16	3·11	3·27
	2	1·40	0·74	0·132	0·54	2·11	2·94	3·07
	3	1·22	0·60	0·119	0·50	2·16	3·17	3·29
Mean*		1·31	0·66	0·140	0·51	2·14	3·07	3·21
Spayed ewes, ODB alone	C	1·86	1·44	—	0·38	0·69	1·13	1·13
	1	1·92	1·01	0·264	0·85	2·33	3·10	3·27
	2	1·70	0·96	0·323	0·55	2·15	3·00	3·20
	3	1·78	0·85	0·397	0·53	2·42	3·80	4·01
Mean*		1·80	0·94	0·328	0·58	2·30	3·30	3·49
Spayed ewes, P alone	C	1·57	1·27	—	0·33	0·79	0·92	0·92
	1	1·35	0·77	0·156	0·69	1·35	2·68	2·74
	2	1·24	0·67	0·061	0·51	1·77	2·94	2·82
	3	1·22	0·69	0·074	0·48	2·03	3·42	3·46
Mean*		1·27	0·71	0·097	0·56	1·72	3·01	3·01
Spayed ewes, no treatment	C	2·08	1·57	—	0·51	1·11	1·03	1·03
	1	2·05	1·40	0·050	0·54	1·43	1·75	1·77
	2	2·09	1·41	0·038	0·65	1·48	1·63	1·64
	3	1·93	1·22	0·030	0·67	1·51	1·62	1·64
Mean*		2·02	1·34	0·039	0·62	1·48	1·62	1·68

*Mean does not include saline control.

TABLE 5

SUMMARY OF THE ANALYSES OF VARIANCE OF THE DATA IN TABLE 3

ODB = oestradiol benzoate; P = progesterone

Source of Variation	Degrees of Freedom	Total Oxygen Uptake	Oxygen Uptake due to Glucose Oxidation	Oxygen Uptake due to Lactate Oxidation	Other Oxygen Uptake	Glucose Utilized	Lactate Accumulated	Lactate Formed
Main effects								
Between treatments								
Normal *v.* rest	1	3·71	0·25	0·0051	0·42	0·50	0·50	0·38
Within spayed ewes								
Effect of ODB	1	0·42	0·02	0·0087	0·41	0·02	7·95	7·11
Effect of P	1	2·86	0·63	0·0486	0·29	17·93*	61·08*	58·71*
ODB × P	1	0·65	0·13	0·2636	0·05	1·59	2·57	0·06
Between ewes within treatment	5	4·30	1·07	0·0739**	0·14	4·04	8·20	8·05
Between stages								
Control *v.* rest	1	24·11**	17·77**	—	0·00	40·51**	183·16**	168·33**
Within stages	2	0·46	0·05	0·0090	0·04	0·01	0·47	0·58
Between ejaculates within ewes	10	1·50**	0·48**	0·0105**	0·13**	27·33**	10·00**	9·86**
Interactions								
Stage × ewes								
Stage × treatment	12	0·93	0·22	0·0033	0·08	0·71	1·79	1·81
Stage × ewes within treatment	15	0·76**	0·17**	0·0040*	0·06**	0·45**	1·02	1·03
Ejaculates within ewes × stage	30	0·11	0·04	0·0013†	0·01	0·14**	0·95**	0·94**
Duplicates	80	0·02	—	—	—	0·03	0·09	0·09

*$P < 0·05$. **$P < 0·01$. † 20 degrees of freedom.

TABLE 6

SUMMARY OF ANALYSES OF VARIANCE OF DATA IN TABLE 4

All values are mean squares. ODB = oestradiol benzoate; P = progesterone

Source of Variation	Degrees of Freedom	Total Oxygen Uptake	Oxygen Uptake due to Glucose Oxidation	Oxygen Uptake due to Lactate Oxidation	Other Oxygen Uptake	Glucose Utilized	Lactate Accumulated	Lactate Formed
Main effects								
Between treatments								
Normal *v.* rest	1	14·58*	3·74*	0·02	0·26	27·88**	120·24**	118·27**
Within spayed ewes								
Effect of ODB	1	0·30	0·60	0·33	0·03	6·85	20·11	14·59
Effect of P	1	10·29*	2·67*	0·05	0·10	0·07	4·58	5·28
ODB × P	1	0·51	0·33	0·18	0·00	0·08	11·07	10·68
Between ewes within treatment	5	3·15	0·83	0·10**	0·15	1·81	13·77	13·38
Between stages								
Control *v.* rest	1	0·74	3·54*	—	0·53*	38·06**	134·50**	123·34**
Within stages	2	0·15	0·09	0·02	0·00	0·28	1·30	0·58
Between ejaculates within ewes	10	1·57**	0·50	0·00	0·12**	2·34**	9·69**	9·51**
Interactions								
Stage × ewes								
Stage × treatment	12	0·09	0·03	0·02	0·02	0·77	2·23	2·11
Stage × ewes within treatment	15	0·87**	0·24**	0·01**	0·06**	0·51*	2·18**	2·09**
Ejaculates within ewes × stage	30	0·07**	0·04	0·002†	0·02	0·20**	0·70**	0·68**
Duplicates	80	0·03	—	—	—	0·02	0·06	0·07

*$P < 0·05$. **$P < 0·01$. † 20 degrees of freedom.

IV. Discussion

The effect of fluid from the fallopian tube of normal ewes on the respiratory activity of spermatozoa appeared to be very variable and, overall, respiration was depressed. These results are at variance with those reported for other species. In the cow, Olds and Van Demark (1957) found that tubal fluid enhanced the oxygen uptake of bull spermatozoa. However, in such incubations, where no extra substrate was added to the diluent, this stimulation is probably due to substrates present in the fluid (Wales and Restall 1966).

Hamner and Williams (1963) found that tubal fluid from the rabbit caused an increase in respiratory activity in rabbit spermatozoa and further proposed that this was due to bicarbonate present in the fluids (Hamner and Williams 1964). Ram spermatozoa do not appear to be affected by the presence of metabolic carbon

Table 7

METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUIDS FROM NORMAL EWES AND SPAYED EWES RECEIVING HORMONE TREATMENTS

Values are expressed as a percentage of those obtained in the saline controls

Cycle of Treatment	Type of Ewe	Respiration		Glycolysis		
		Total Oxygen Uptake	Glucose and Lactate Oxidation	Total Glucose Utilized	Lactate Accumulated	Lactate Formed
1	Normal	85·7	63·9	234·0	286·0	294·4
	Spayed	48·1	48·8	194·8	241·0	249·1
2	Normal	95·6	80·8	225·0	264·2	269·7
	Spayed	90·4	77·4	203·2	261·9	271·4

dioxide during incubation (Wales and O'Shea 1966). Bicarbonate was found to be present in all the fluids used in this study (Restall and Wales 1966), but in other experiments the metabolism of spermatozoa was unaffected by the presence or absence of carbon dioxide when incubated in the genital fluids of the ewe (Wales and Restall 1966). Because of the differences in methods of collection of fluid and in experimental technique, comparisons of the two studies must be made cautiously but a true species difference may exist.

The most consistent effect of the tubal fluid from the normal ewes was the stimulation of glycolysis, and generally glucose breakdown was twice that of the saline controls. The factor responsible for this stimulation is not known. The absence of any difference in the effects of fluids from various stages of the oestrous cycle indicates that, metabolically, spermatozoa could tolerate the tubal environment for long periods. The significant differences between ewes in the amount of lactate oxidized probably reflects the varying initial levels of this substrate in the tubal fluids (Restall and Wales 1966).

The effects observed in fluids from the spayed ewes are essentially similar to those observed in the normal ewes. The reduction in glycolysis in the first cycle and in respiration in the second cycle in fluids from ewes receiving progesterone is un-

explained, but it may be noted that progesterone alters the levels of protein, carbohydrate, and lactate in the fluids, the latter two differing between cycles of treatment (Restall and Wales 1966). In general, the hormone treatments used did not affect the metabolism of spermatozoa incubated in the derived fluids.

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