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EFFECT OF THE FEMALE REPRODUCTIVE TRACT ON SPERM METABOLISM IN THE RABBIT AND FOWL

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Summary. A fourfold increase in oxygen uptake by rabbit spermatozoa incubated in the rabbit uterus and a twofold increase in cock spermatozoa incubated in the hen's oviduct over ejaculated spermatozoa is the first reproducible biochemical change to be demonstrated in spermatozoa residing in the female genital tract. It was shown that these rapidly respiring spermatozoa were capacitated because they fertilized 18 % of the eggs tested as compared to no eggs fertilized by freshly ejaculated spermatozoa.

It was found that oviduct fluid *in vitro* stimulates respiration fivefold in spermatozoa protected from light. Increase in oxygen consumption by spermatozoa residing in the female reproductive tract may be an important part of the capacitation process.

INTRODUCTION

The discovery by Austin (1951) and Chang (1951) that rat or rabbit spermatozoa were unable to fertilize ova until after an incubation period in the female reproductive tract opened up a new field of investigation, i.e. the effect of the female tract on spermatozoa. The change that spermatozoa undergo in the female tract has been termed 'capacitation' by Austin (1952) and its existence has been confirmed by Hadek (1959) and Noyes (1960).

In view of these discoveries, the present investigation was undertaken on the biochemical effects of the female reproductive tract on spermatozoa.

MATERIALS AND METHODS

Semen was collected from rabbits with an artificial vagina and the spermatozoa washed free of seminal plasma as described by Lardy & Phillips (1943). Aliquots of semen were accurately measured into centrifuge tubes and diluted with two to three volumes of calcium-free Krebs'-Ringer phosphate solution at pH 7·0. The diluted semen was then centrifuged at 142 *g* for 8 min. The supernatant solution was then removed and the spermatozoa resuspended in calcium-free Krebs'-Ringer phosphate. This washing procedure was repeated two times. Then 2·5 mg of streptomycin was added per ml of resuspended washed spermatozoa. Some of the spermatozoa were used for respiration

studies and referred to as fresh, washed spermatozoa, and the remainder of the spermatozoa were used for in-utero incubation studies and for incubation *in vitro* in a water bath at 37° C. Avian spermatozoa used in these studies were collected by a method described by Burrows & Quinn (1937). The avian spermatozoa were washed in the same way as rabbit spermatozoa except that they were centrifuged at 163 *g* for 8 min and resuspended in avian Ringer's or Tyrode's solution. The respiration studies on the fresh washed spermatozoa were started within 1 hr after collection since it was found that further delay resulted in a considerable drop in respiration rate. All respiration studies were carried out in a Warburg constant volume respirometer.

Isolation of the rabbit uterus for incubation of spermatozoa *in utero* was carried out in the following manner. Nembutal (Abbott Laboratories) 1 grain per 5 lb body wt was used as an anaesthetic, a mid-abdominal midline incision was made and the uterus brought up through the incision. The uteri were then ligated just anterior to the cervix avoiding the blood supply to the uterus through the broad ligament by placing the ligature between the vessels.

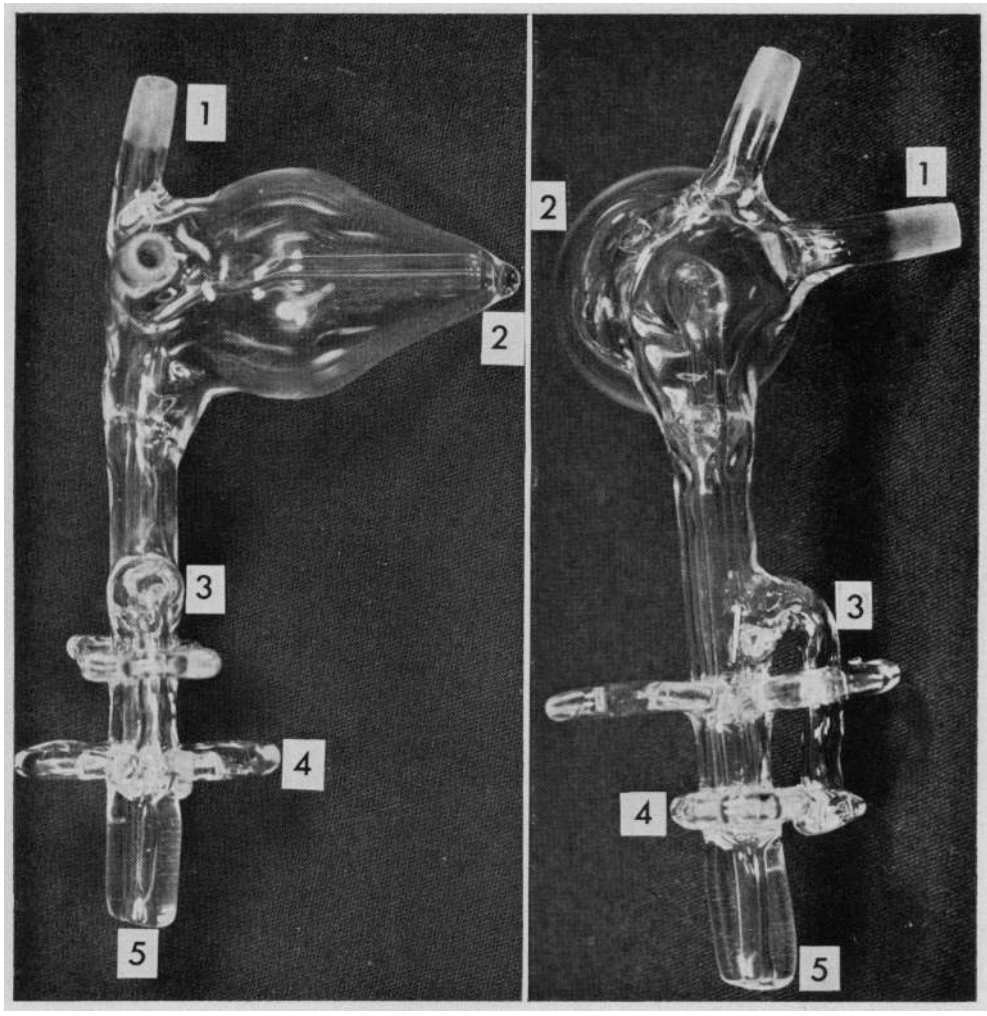
Spermatozoa were injected through a 22- or 24-gauge needle into the lumen of the uterus anterior to the ligature. The uterus was then returned to the abdominal cavity and the incision closed.

After the spermatozoa had been incubated the desired length of time, the doe was killed and both uteri removed. The uteri were flushed out with calcium-free Krebs'-Ringer phosphate solution to obtain the in-utero incubated spermatozoa. If debris was present in the washings, it was allowed to settle for 3 to 5 min and washings were then decanted through a double layer of cheesecloth. Following this the spermatozoa were washed as previously described. These spermatozoa were then used for studying the respiration of in-utero incubated spermatozoa and for capacitation studies as described by Chang (1951).

In isolating a segment of the hen oviduct for sperm incubation studies *in vivo*, nembutal or ether was used as an anaesthetic. A longitudinal incision was made on the left lateral posterior abdominal region through a fat layer to expose the oviduct. The oviduct was brought out through the incision and a segment ligated taking care to avoid the blood supply. Spermatozoa were injected into the isolated segment and the remainder of the procedure was the same as described for the rabbit.

The method of collection of rabbit oviduct fluid used during this investigation was a modification of a method described by Clewe & Mastroianni (1960) incorporating several new and advantageous features. Polyethylene tubing was connected from both oviducts to a flask inside the abdominal cavity which had a tube opening to the exterior. An air inlet is attached to the side of the exterior tube to allow sterile air to enter replacing the fluid withdrawn. Collection of the fluid was achieved by attaching a syringe to the glass tubing which extends through the exterior tube down to the bottom of the collecting flask. Collection of 1 to 2·5 ml per day per rabbit is achieved with this method. Cottonwool plugs soaked in a streptomycin solution, dried and autoclaved, are inserted into the exterior tube and the air inlet tube to maintain sterile conditions in the collecting flask. A photograph of the flask is shown in Plate 1.

These modifications have the advantage of keeping the oviduct fluid at



Oviduct fluid collecting flask. Left: Lateral view; Right: Dorsal view. 1. Tubes for connecting oviducts to flask via polyethylene tubing. 2. Collecting flask. 3. Air inlet tube. 4. Flanges at right angles to each other; the abdominal wall is sutured between the flanges. 5. Outlet tube for withdrawing oviduct fluid.

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body temperature and away from light. The flask being on the inside of the rabbit with only a small opening in the skin is not irritating and no tubing or collecting apparatus is exposed for the animal to molest. Biting and clawing at the tube and collecting apparatus was a major problem with our attempts to use the technique described by Clewe & Mastroianni (1960).

Infiltration of leucocytes into the uterus of the rabbit during in-utero incubation of spermatozoa occurred and consistently complicated recovery of spermatozoa. Leucocytes always remained after washing the spermatozoa free of the female tract secretions. The amount of oxygen utilized by the leucocytes was determined and subtracted from the total oxygen uptake. The oxygen uptake by the leucocytes was determined by incubating heat-killed spermatozoa (65° C for 10 min) in one uterus of the rabbit and fresh, washed spermatozoa in the other uterus. After incubation, the spermatozoa were recovered from each uterus, washed, and both spermatozoa and leucocytes counted. Then respiration studies were carried out. All the oxygen taken up by the heat-killed spermatozoa plus leucocytes was attributed to the leucocytes. The oxygen uptake by the leucocytes was then subtracted from the total oxygen uptake by the live fresh, washed, in-utero incubated spermatozoa plus leucocytes to determine the amount of oxygen taken up by the spermatozoa.

Lactic acid formation by the spermatozoa was determined by the Barker & Summerson (1941) method.

It was found that rabbit oviduct fluid took up oxygen, therefore a control flask containing 1 ml of oviduct fluid was included whenever the effect of oviduct fluid on respiration of spermatozoa was studied.

RESULTS

STIMULATION OF SPERM RESPIRATION BY INCUBATION IN THE FEMALE REPRODUCTIVE TRACT

The effect of length of time of incubation in the female tract on sperm respiration was studied in the rabbit. Results in Table 1 show that the respiration rate increases with length of incubation time up to 6 hr and then drops off by the 7th hour.

A determination of the amount of oxygen utilized by leucocytes which contaminated the sperm samples taken from the rabbit's uterus is shown in Table 2. After washing the spermatozoa free of the female tract secretions, the sample contained $0{\cdot}07$ to $0{\cdot}25 \times 10^8$ leucocytes per 10^8 spermatozoa. It was found that 21 µl of oxygen per hour was taken up by 10^8 leucocytes.

Table 3 shows that incubation of cock spermatozoa for 2 hr in the section of the hen's oviduct adjacent to the infundibulum (magnum or albumen-secreting portion) caused a 2·5-fold increase in the respiration over that for fresh ejaculated washed spermatozoa; leucocyte infiltration into the oviduct of the hen during incubation of spermatozoa did not occur.

It was found that rabbit spermatozoa incubated *in utero* produced slightly more lactic acid than freshly ejaculated washed spermatozoa did. These results are shown in Table 4.

TABLE 1

EFFECT OF IN-UTERO INCUBATION TIME ON RABBIT SPERM RESPIRATION

Experiment No.	Z_{O_2}*						
	Freshly washed spermatozoa	In-vitro incubation 6 hr	In-utero incubation†				
			2 hr	4 hr	5 hr	6 hr	7 hr
165	12·8		5·7	14·5			
172	6·3			19·4		47·5	
152	3·6				23·6		
175	5·0				23·0		
48	8·7	8·5				23·6	
49	12·0	7·4				19·9	
57	4·0	5·1				19·2	
59	6·5					30·8	
81	3·8					27·1	
157	1·7					42·0	
74	7·4	7·3					13·0
61	6·2						6·6
97	4·3						26·8
Average	6·5	7·2	5·7	16·8	23·3	30·0	15·8

* Z_{O_2} values (µl oxygen taken up per hour per 10^8 spermatozoa).
† Z_{O_2} values corrected for uptake by leucocytes.

TABLE 2

DETERMINATION OF OXYGEN UPTAKE BY LEUCOCYTES

Spermatozoa treatment	Oxygen (µl/flask)	Z_{O_2}	
		Spermatozoa	Leucocytes
Freshly washed	28·2	6·8	
Heat killed (65° C for 10 min)	0·0	0·0	
Live, in-utero incubation with leucocytes	34·7		
Killed, in-utero incubation with leucocytes	10·5		21·0
Live, in-utero incubation corrected for leucocytes	24·2	14·3	

Oxygen uptake first hour (average of two experiments). In-utero incubation 5 hr.
Concentration of leucocytes was $0{\cdot}50 \times 10^8$ per Warburg flask.
Concentration of spermatozoa was $1{\cdot}69 \times 10^8$ per Warburg flask.

TABLE 3

INCUBATION OF COCK SPERMATOZOA IN OVIDUCT OF HEN

Spermatozoa treatment	Experiment No.					
	15	44	65	68	156	Average
Freshly washed	8·8*	4·3	6·5	5·8	9·6	7·0
In-vitro incubation	6·9	1·0	8·6		2·5	4·8
In-oviduct incubation (2 hr) (Albumen-secreting portion)						
Rewashed	15·7	13·2	29·4	9·1	12·5	16·0
Unwashed			35·5	12·4	17·1	21·8

* Z_{O_2} values (µl oxygen taken up per hour per 10^8 spermatozoa).

EFFECT OF OVIDUCT FLUID ON SPERM RESPIRATION

The true effect of oviduct fluid on sperm respiration can only be fully appreciated when the spermatozoa are protected from light at all times during collection, handling and respiration studies to eliminate the stimulation of

TABLE 4

LACTIC ACID FORMED BY RABBIT SPERMATOZOA

Spermatozoa treatment	*No glucose*	*With glucose*
Freshly washed	0* (4)†	99 (6)
In-utero incubation (6 hr)	0 (6)	110 (6)

* μg lactic acid per hour per 10^8 spermatozoa.
† Number of experiments in parentheses.

respiration by light demonstrated by Hamner & Williams (1961). In Table 5 it is shown that oviduct fluid stimulated a 1·5-fold increase in oxygen uptake in room light compared to a fivefold increase in the oxygen uptake in absence of light. Since spermatozoa are protected from light under natural conditions of reproduction, it appears that metabolic studies on spermatozoa should be conducted on spermatozoa protected from light.

TABLE 5

EFFECT OF OVIDUCT FLUID ON SPERM RESPIRATION

Addition to fresh washed spermatozoa	Q_{O_2}		*No. experiments*
	Dark	*Light*	
Glucose	2·0	7·7	9
Oviduct fluid	8·8	8·8	2
Oviduct fluid and glucose	10·8	11·4	8

TABLE 6

POSSIBLE RELATION OF OXYGEN UTILIZATION TO CAPACITATION

Spermatozoa treatment	Q_{O_2}	*Capacitation studies*	
		No. eggs recovered	*Eggs undergoing division* (%)
Freshly washed	6·3	36	0
In-utero incubation (5 hr)	28·3	51	18

Average for five experiments.

POSSIBLE RELATION OF INCREASED OXYGEN UTILIZATION TO CAPACITATION

An attempt to relate the increased rate of respiration of spermatozoa incubated *in utero* with capacitation has shown that some of the rapidly respiring spermatozoa are capacitated. In Table 6 results show that none of the eggs was fertilized by the slowly respiring freshly washed spermatozoa, while 18 % of

the eggs were fertilized by the rapidly respiring spermatozoa which had been incubated *in utero*. The spermatozoa were deposited in the Fallopian tubes of does which had ovulated 2 hr earlier.

DISCUSSION

The fourfold increase in the respiration rate of rabbit spermatozoa incubated in the doe's uterus and the twofold increase in respiration rate of cock spermatozoa incubated in the hen's oviduct represents the first reproducible biochemical change observed in spermatozoa while residing in the female genital tract. This increase in respiration of the spermatozoa is a true change in the cell itself, since the spermatozoa are thoroughly washed free of the reproductive tract secretions and returned to the same physiological medium as freshly washed spermatozoa for respiration studies.

The changes in spermatozoa directly attributable to capacitation have not yet been found, although several possibilities have been suggested. Austin & Bishop (1958) observed that the acrosome was removed from the head of the spermatozoa before entry into the egg. They suggested that removal of the acrosome may be the mechanism of capacitation. Chang & Slechta (1959) have shown that freshly ejaculated rabbit and guinea-pig spermatozoa had as many acrosomes removed as those recovered after in-utero incubation. They used histochemical techniques, such as tests for acid and alkaline phosphatase, glycogen, lipid, calcium, deoxyribonucleic acid and ribonucleic acid, but could not find any differences by ordinary visual examination between ejaculated spermatozoa and spermatozoa recovered from the uterus. Chang & Thornsteinsson (1959) employed a microspectrophotometric method, and found unexpectedly that there was an increase of deoxyribonucleic acid per cell as shown by feulgen staining of the spermatozoa recovered from Fallopian tubes or from the uterus as compared with freshly ejaculated rabbit spermatozoa.

A possible relationship between the increased oxygen uptake of the spermatozoa and capacitation is indicated by the progressive increase in oxygen utilization by rabbit spermatozoa with increasing time of incubation in the doe's uterus which parallels capacitation data reported by Chang (1951). He found that freshly ejaculated spermatozoa deposited in the Fallopian tubes 2 hr before ovulation fertilized none of the eggs, but spermatozoa deposited at 4 and 6 hr before ovulation fertilized 6 % and 78 % of the eggs, respectively. Another possible relation between the increased oxygen uptake by spermatozoa and capacitation is suggested by the observation that 18 % of the eggs in contact with the rapidly respiring spermatozoa were fertilized as compared with none fertilized in contact with freshly washed spermatozoa. Chang (1951) demonstrated 13 % fertilization in eggs with spermatozoa incubated *in utero* for 5 hr.

Bishop (1956) determined that the interior of the rabbit female tract is aerobic. The oxygen tension is equivalent to about 40 mm Hg in both the fluids and mucosa of the oviduct and uterus. Thus, sperm respiration can be supported in both the uterus and oviduct.

The possible role of leucocytes in sterility as suggested by Austin (1960) was re-emphasized by the observation that leucocyte infiltration usually occurred after the 3rd hour of incubation of spermatozoa in the rabbit uterus. Spermatozoa in large numbers were found surrounding each leucocyte, adhering to the leucocytes by the anterior end of the head yet maintaining fully active motion of their tails. Many spermatozoa are lost during the washing procedure after recovery from the uterus; therefore, high sperm concentrations (20×10^8 cells) have to be injected into the uterus in order to recover 2 to 3×10^8 spermatozoa after 5 to 6 hr incubation. These high sperm concentrations are much above that which would normally be considered physiological. Under these conditions, it would not be expected that all the spermatozoa would be capacitated, and this may explain why only 18 % of the eggs were fertilized by the spermatozoa incubated *in utero*.

The uptake of 21 µl of oxygen per hour per 10^8 cells by the leucocytes compares favourably with Krines & Strauss's (1961) observation that human leucocytes take up 28 µl oxygen per hour per 10^8 cells while phagocytizing latex particles *in vitro*.

Hamner & Williams (1961) showed that spermatozoa of rabbit, chicken, and man failed to respire, or respired at a very low rate, when completely protected from all light during collection, handling and respiration studies. Spermatozoa similarly protected from light, incubated *in utero*, and protected from light on recovery from the uterus, respired at a rate similar to that of spermatozoa exposed to light before and after incubation *in utero*.

A survey by Hamner (1962) of the effect of many metabolically active compounds such as sugars, nucleic acids, nucleosides, coenzymes, growth factors, and enriched medium for lactic acid bacteria showed that addition of these substances to calcium-free Krebs'-Ringer phosphate with glucose gave no increase in oxygen uptake above that obtained with glucose alone. The observation of an increase in oxygen uptake from addition of oviduct fluid while none of the above compounds was effective, suggests that an unknown stimulating factor may be present in oviduct fluid. The results of two experiments show that oviduct fluid alone is not as stimulating as oviduct fluid with glucose which suggests that exogenous respiration is being affected rather than endogenous respiration.

Bishop (1957) has shown that oviduct fluid contains small amounts of lactate, fructose, glucose and phospholipids. Because of the stimulating effect of added glucose on spermatozoa incubated *in vitro*, it appears unlikely that such energy sources are involved.

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