EFFECT OF INCUBATION CONDITIONS ON THE METABOLIC RESPONSE OF RAM SPERMATOZOA IN THE PRESENCE OF FLUIDS FROM THE GENITAL TRACT OF THE EWE

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Summary

Modifications to the incubation conditions used to study the effect of genital fluids on the metabolism of ram spermatozoa have been shown to affect the parameters measured.

When tubal fluid is diluted up to 1 in 16 in the incubation mixture, the usual depression of spermatozoal respiration disappears. Enhancement of glycolysis, however, is unaffected by dilution of the tubal fluid. Greater differences between the respiration of spermatozoa in saline controls and in tubal fluid are observed if the concentration of spermatozoa is high rather than low. The reverse is true for glycolysis in the spermatozoa. The buffer system used in the incubation further modifies the parameters measured. When compared with a phosphate buffer system, bicarbonate buffer appears to enhance glycolysis by spermatozoa in saline, and depresses it in spermatozoa incubated in uterine and tubal fluids, thus decreasing the magnitude of the stimulation due to the genital fluid.

The results obtained are consistent in so far as a depression of respiration and stimulation of glycolysis in ram spermatozoa occurred whenever significant amounts of genital fluid were present.

I. INTRODUCTION

Many recent reports have examined the effect of fluids from the female reproductive tract on the metabolism of spermatozoa (Olds and Vandemark 1957; Hamner and Williams 1963, 1964; Kirton and Hafs 1965; Restall and Wales 1966; Wales and Restall 1966; Foley and Williams 1967; Murdoch and White 1968). The effects reported have varied in some cases. For instance, Restall and Wales (1966) using fluid from the oviduct of the ewe found that generally the fluid depressed oxygen uptake of spermatozoa but enhanced glycolysis. Hamner and Williams (1963, 1964) and Murdoch and White (1968) using oviduct fluid from the rabbit have found that the oxygen uptake of spermatozoa is enhanced by the fluid and have proposed that the stimulation is due to the bicarbonate content of the fluid. Wales and Restall (1966) could not demonstrate any effect of metabolic bicarbonate on the respiration of ram spermatozoa incubated in genital tract fluids.

While species differences are likely to occur, other factors may be responsible for the differences in these studies. For example the conditions under which metabolic studies are carried out vary from laboratory to laboratory thus making comparisons difficult.

This paper reports some experiments designed to study the effect of varying incubation conditions on the metabolic response of ram spermatozoa incubated in fluids from the genital tract of the ewe.

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II. Materials and Methods

(a) *Genital Fluids*

Fluids secreted by the fallopian tubes were collected as previously described (Restall 1966). In all experiments the sample of oviduct fluid was obtained by pooling collections from at least four ewes, although fluids from different ewes were used in different experiments. Cervicovaginal fluid was obtained by aspiration from the vaginae of ewes in oestrus and pooled before use. Uterine fluid was collected from ewes with cannulated uteri and pooled samples from at least four ewes were used.

(b) *Semen*

Ram semen was collected by electrical stimulation and only ejaculates with good initial motility were used. After collection, the spermatozoa were washed twice in the appropriate isotonic buffer–saline diluent. 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 except where specified.

(c) *Incubation of Semen*

Five experiments are reported. In the first four, spermatozoa were incubated at 37°C in standard Warburg flasks in the presence of one of the genital fluids. Flasks were made up with aliquots of oviduct fluid (considered to be isotonic) and sufficient incubation diluent to give a final volume of 1·0 ml.

The incubation diluents consisted of buffer at the required molar concentration, substrate, and sufficient sodium chloride to make the particular diluent isotonic. Incubation diluents were prepared for each experiment and, where bicarbonate buffer was used, a particular diluent was prepared for each genital fluid in accordance with the measured bicarbonate content of that genital fluid. In each case tonicity was maintained by addition of the required amount of sodium chloride. Thus, no further adjustments to tonicity were required when all the contents of each Warburg flask were placed together. Control flasks had the genital fluid replaced by an equal volume of 0·9% (w/v) sodium chloride. In the fifth experiment, incubation was carried out in small test tubes in a water-bath at 37°C, with those tubes containing bicarbonate being maintained under an atmosphere of 5% carbon dioxide in air. Precise experimental details are given in Section III.

(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 content of the extracts were estimated by enzymic methods (Barker and Britton 1957; Huggett and Nixon 1957) and fructose content by the method of Mann (1948) as modified by White (1959).

(e) *Statistical Analyses*

All results have been subject to standard analyses of variance, which are presented in summary form in the tables, giving only the number of degrees of freedom and the variance ratios for each source of variation. In all cases the mean square for ejaculate interactions has been used for tests of significance.

III. Experimental Details and Results

(a) *Experiment 1: Effect of Dilution of Oviduct Fluid on the Metabolism of Spermatozoa*

Three samples of oviduct fluid were added to Warburg flasks to give a final concentration of 1:2, 1:4, 1:8, or 1:16 of oviduct fluid. Each Warburg flask contained an aliquot of oviduct fluid, 20 mM phosphate buffer (pH 7·2), 10 μmoles of glucose, and 0·3 ml of spermatozoal suspension. Duplicate flasks were prepared,

0·1 ml of [U-^{14}C]glucose being added to one and 0·1 ml of [1-^{14}C]lactate as carrier-free isotope in 0·9% (w/v) sodium chloride to the other. The oxidation of these two substrates was calculated by radioassay of the trapped carbon dioxide and measurement of the specific activity of the two substrates as previously described (Restall and Wales 1966). Two ejaculates were used with each sample of oviduct fluid and the flasks were incubated for 3 hr at 37°C.

TABLE 1

METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUID AT VARIOUS DILUTIONS

All values are in μmoles/10^8 cells over the experimental period (3 hr), except where otherwise stated. The data are means of two ejaculates in three samples of oviduct fluid

Dilution of Fluid	Total Oxygen Uptake	Carbon Oxidized (μg-atoms)	Glucose Utilized	Lactate Accumulated
Control (saline)	2·23	1·79	1·32	1·49
1:2	1·12	0·59	3·66	7·08
1:4	1·52	1·02	3·37	5·82
1:8	2·07	1·60	2·81	4·33
1:16	2·67	2·09	2·38	3·32

Summary of the analyses of variance

Source of Variation	Degrees of Freedom	Variance Ratios: Total Oxygen Uptake	Carbon Oxidized (μg-atoms)	Glucose Utilized	Lactate Accumulated
Effect of dilution (A)	(4)				
Control *v.* others	1	1·94	4·16	116·83**	125·88**
Within dilutions					
Linear	1	22·37**	30·84**	46·96**	96·02**
Quadratic	1	0·18	0·00	0·21	0·18
Cubic	1	0·01	0·08	0·42	0·31
Effect of fluid sample (B)	2	20·36**	14·47**	28·87**	18·01**
$A \times B$	8	9·27**	3·82*	1·70	2·35
Ejaculate difference within fluid samples (C)	3	25·11**	10·65**	13·13**	7·69**
$A \times C$ (mean square)	12	*0·0782*	*0·0653*	*0·1457*	*0·4330*
Duplicates (mean square)	30	*0·0405*		*0·1156*	*0·0854*

* $P < 0·05$. ** $P < 0·01$.

The results of this experiment are given in Table 1 together with a summary of the analyses of variance. These results show that at the higher concentrations of oviduct fluid the oxidation of substrates by the spermatozoa was depressed, but that the effect disappeared as the fluid was diluted. On the other hand glycolysis was always enhanced, as compared with the controls, even at a dilution of 1:16. Overall differences were found between samples of oviduct fluid but the responses were linear in all cases. A dilution by fluid interaction was present for the oxidative

parameters, two samples of fluid having a higher oxidative response than the control at the 1:16 dilution, the other being below the control.

The glycolytic response was linear over the range of dilution and at all dilutions of fluid was higher than in control incubations.

(*b*) *Experiment 2: Effect of Dilution of Spermatozoa on their Metabolism in the Presence of Oviduct Fluid*

Washed spermatozoa from two ejaculates were added to Warburg flasks such that a final concentration of either approximately 1×10^8 or 4×10^8 cells per flask was obtained. Each Warburg flask contained 0·3 ml of oviduct fluid, 20 mM phosphate buffer, 10 μmoles of glucose, and 0·3 ml of spermatozoal suspension. Duplicate flasks were prepared.

TABLE 2

EFFECT OF TUBAL FLUID ON THE METABOLISM OF SPERMATOZOA AT DIFFERENT CONCENTRATIONS

All values are expressed as μmoles per 10^8 cells over the experimental period (3 hr). The data are means for two ejaculates in each treatment

Type of Fluid	Concentration of Spermatozoa per Flask	Total Oxygen Uptake	Glucose Utilized	Lactate Accumulated
Saline (control)	1×10^8	1·30	0·78	0·92
	4×10^8	1·90	0·99	1·61
Tubal fluid	1×10^8	0·67	2·53	3·72
	4×10^8	0·38	1·99	3·56

Summary of the analyses of variance

Source of Variation	Degrees of Freedom	Variance Ratios		
		Total Oxygen Uptake	Glucose Utilization	Lactate Accumulated
Effect of concentration of spermatozoa (*A*)	1	2·71	0·68	1·75
Effect of fluid, saline *v.* tubal fluid (*B*)	1	125·71**	49·37**	139·42**
Ejaculate differences (*C*)	1	2·63	3·11	7·44
$A \times B$	1	21·88	13·83*	4·59
Ejaculate interactions (mean square)	3	*0·0366*	*0·0415*	*0·1620*
Duplicates (mean square)	8	*0·0315*	*0·1540*	*0·0459*

* $P < 0·05$. ** $P < 0·01$.

The results are shown in Table 2 together with a summary of the analyses of variance. Again the results show a depression of oxygen uptake and an enhancement of glycolysis when oviduct fluid was present. In flasks containing oviduct fluid, both oxygen uptake and glycolysis were greater at the dilute spermatozoal concentration. On the other hand, in control flasks, the metabolic response of the spermatozoa was greatest at the high concentration of spermatozoa.

(c) *Experiment 3: Effect of Phosphate, Tris, or Veronal Buffer on the Metabolism of Spermatozoa Incubated in Oviduct Fluid*

Ejaculates of semen were divided into three portions and each portion washed with either phosphate, Tris, or veronal buffer at pH 7·2. The washed spermatozoa ($1–2 \times 10^8$ cells per flask) were added to Warburg flasks containing 0·3 ml of oviduct fluid, 10 μmoles of glucose, and the appropriate buffer to a final volume of 1·0 ml. The final concentration of buffer in each flask was 20 mM. Five ejaculates were used representing five replications of the experiment.

TABLE 3

EFFECT OF PHOSPHATE, TRIS, OR VERONAL BUFFER ON THE METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUID

All values are expressed as μmoles per 10^8 cells over the experimental period (3 hr). The data are means of five ejaculates

Type of Buffer	Type of Fluid	Total Oxygen Uptake	Glucose Utilized	Lactate Accumulated
Phosphate	Saline	1·40	0·88	1·18
	Tubal fluid	0·90	2·23	4·78
Tris	Saline	1·80	0·81	0·72
	Tubal fluid	0·81	2·06	3·71
Veronal	Saline	0·74	0·97	1·86
	Tubal fluid	0·31	2·31	4·98

Summary of the analyses of variance

Source of Variation	Degrees of Freedom	Variance Ratios		
		Total Oxygen Uptake	Glucose Utilized	Lactate Accumulated
Effect of buffers (A)	2	119·79**	1·61	27·41**
Effect of fluids (B)	1	213·71**	192·82**	578·59**
Ejaculate differences (C)	4	3·95*	8·21**	6·60*
$A \times B$	2	13·29**	0·10	1·91
Ejaculate interactions (mean square)	20	*0·0289*	*0·1300*	*0·3094*
Duplicates (mean square)	30	*0·0047*	*0·0335*	*0·0382*

* $P < 0·05$. ** $P < 0·01$.

The results (Table 3) show overall effects similar to those noted previously when spermatozoa were incubated in tubal fluid. However, the oxygen uptake of the spermatozoa in the control flasks was highest in Tris and lowest in veronal buffer. In the presence of oviduct fluid this ranking was altered, the uptake of oxygen by spermatozoa in phosphate and Tris buffers being more nearly equal. While the amount of glucose utilized by the spermatozoa was not affected significantly by the type of buffer used, significant differences were apparent in the amount of lactate accumulating (veronal = phosphate > Tris).

(*d*) *Experiment 4: Effect of Fructose and of Glucose on the Metabolism of Spermatozoa Incubated in Oviduct Fluid*

Aliquots of washed spermatozoal suspension were incubated in Warburg flasks for 3 hr under aerobic and anaerobic conditions in the presence of oviduct fluid in 20 mM phosphate buffer (pH 7·2) with either glucose or fructose as the added substrate. For anaerobic conditions, flasks were flushed with dry nitrogen for 10 min

TABLE 4

EFFECT OF FRUCTOSE AND OF GLUCOSE ON THE METABOLISM OF SPERMATOZOA INCUBATED IN TUBAL FLUID IN AEROBIC AND ANAEROBIC CONDITIONS

All values are expressed as μmoles per 10^8 cells over the experimental period (3 hr). The data are means of two ejaculates in duplicate incubations

Substrate	Type of Fluid	Aerobic			Anaerobic	
		Total Oxygen Uptake	Carbohydrate Utilized	Lactate Accumulated	Carbohydrate Utilized	Lactate Accumulated
Fructose	Saline	2·37	1·04	1·75	2·09	4·53
	Fluid	0·83	5·00	10·12	4·44	10·32
Glucose	Saline	2·74	0·69	1·53	2·03	4·14
	Fluid	0·91	4·89	10·31	4·61	10·06

Summary of the analyses of variance

Source of Variation	Degrees of Freedom	Variance Ratios		
		Total Oxygen Uptake	Carbohydrate Utilized	Lactate Accumulated
Effect of substrate (*A*)	1	0·21	0·34	0·31
Effect of gas phase (*B*)	1	—	6·53*	19·54
Saline *v.* fluid (*C*)	1	11·36**	469·14**	572·95**
Ejaculate difference (*D*)	1	1·12	32·16**	36·69**
$A \times B$	1	—	0·87	0·27
$A \times C$	1	0·09	0·60	0·19
$B \times C$	1	—	28·96**	20·40
Ejaculate interactions (mean square)	8	*0·2033*†	*0·1832*	*0·7264*
Duplicates (mean square)	16	*0·9385*‡	*0·1001*	*0·2029*

* $P < 0·05$. ** $P < 0·01$. † 3 degrees of freedom. ‡ 8 degrees of freedom.

at the start of incubation. The flasks, of 5 ml capacity, contained 0·3 ml of spermatozoal suspension ($1–2 \times 10^8$ cells per flask), 0·3 ml of oviduct fluid, and 0·2 ml of an isotonic diluent containing 10 μmoles of the substrate. Duplicate flasks were prepared and two ejaculates were used as replications.

This experiment again demonstrated the depression of oxygen uptake and stimulation of glycolysis on spermatozoa when incubated in oviduct fluid (Table 4). The type of substrate used, fructose or glucose, had no effect on the results.

In the saline controls the amount of carbohydrate utilized was considerably higher under anaerobic conditions than under aerobic conditions. However, in the presence of oviduct fluid this Pasteur effect was abolished.

(e) *Experiment 5: Effect of Phosphate or Bicarbonate Buffer on Glycolysis of Spermatozoa Incubated in Fluids from the Genital Tract*

Incubation of washed spermatozoal suspensions was carried out in small test tubes containing 0·3 ml of either cervicovaginal fluid, uterine fluid, or oviduct fluid in 1·0 ml of either isotonic phosphate buffer (20 mM, pH 7·3) or bicarbonate buffer (13·5 mM, pH 7·3) at 37°C for 6 hr.

TABLE 5

UTILIZATION OF GLUCOSE BY SPERMATOZOA IN PHOSPHATE AND BICARBONATE BUFFER INCUBATED WITH FLUIDS FROM THE GENITAL TRACT OF THE EWE

The data are means of seven ejaculates. The values are expressed as μmoles per 10^8 cells over the experimental period (6 hr)

Genital Tract Fluid	Glucose Utilized	Summary of the Analysis of Variance		
		Source of Variation	Degrees of Freedom	Variance Ratios
Phosphate buffer				
Saline (control)	0·89	Effect of buffer (*A*)	1	1·08
Cervicovaginal	3·16	Effect of genital fluid (*B*)	3	33·07**
Uterine	5·29	Ejaculate differences (*C*)	6	9·98**
Tubal	6·00	*A* × *B*	3	8·56**
Bicarbonate buffer		Ejaculate interactions		
Saline (control)	2·76	(error mean square)	42	*1·0895*
Cervicovaginal	4·64			
Uterine	3·84			
Tubal	5·26			

** $P < 0·01$.

The bicarbonate content of the tract fluids was determined manometrically and the final concentration of bicarbonate in the incubation mixture adjusted to 0·0135M. Tubes containing bicarbonate buffer were incubated in the presence of a gas phase containing 5% carbon dioxide. Each tube contained 15 μmoles of glucose. Glucose utilization was determined at the end of the 6-hr incubation period. Seven ejaculates of ram semen were used, each being split and washed in the appropriate buffer before being added to the incubation mixture. The final concentration of spermatozoa was $1–2 \times 10^8$ cells per tube in a volume of 1·0 ml.

The results are summarized in Table 5. All the genital tract fluids were observed to enhance glucose utilization when compared with the controls, oviduct fluid having the greatest effect. However, the type of buffer used modified the magnitude, but not the direction of this effect. In flasks containing bicarbonate buffer, glycolysis was enhanced in the saline controls and in cervicovaginal fluid while being depressed in uterine and tubal fluids when compared with phosphate buffer.

IV. Discussion

In earlier reports (Wales and Restall 1966) incubations were performed with phosphate as the buffer, with a 1:3 dilution of genital fluid, a spermatozoal concentration of $1–2 \times 10^8$ cells per flask, and glucose as the added substrate. The experiments described in this paper show that the magnitude of the metabolic response can vary if some of the incubation conditions are changed.

Altering the concentration of spermatozoa had significant effects. With high concentrations a greater difference between oxygen uptake in saline and in tubal fluid was observed than if lower concentrations were used. In the case of glycolysis, a greater difference between saline and tubal fluid was observed with low concentrations of spermatozoa. Similar effects have been noted elsewhere. O'Shea and Wales (1965), using sorbitol as the sole substrate, observed that an increase in concentration resulted in increased oxidative metabolism and a decreased anaerobic sorbitol dismutation. With fructose as substrate, Wales and O'Shea (1966) noted higher oxygen uptake with increased spermatozoal concentration.

Hamner and Williams (1964) showed a maximum stimulation of respiration of spermatozoa incubated in oviduct fluid from the rabbit when the fluid was diluted 1:6. At higher dilutions the stimulation was sharply reduced. Murdoch and White (1968), however, found a linear response of oxygen uptake of rabbit spermatozoa in tubal fluid over a dilution range from 1:3 to 1:9. While oxygen uptake was variable in the present experiments, glycolysis was always enhanced in tubal fluid, even at dilutions of 1:16. As these experiments were carried out in the presence of potassium hydroxide, the effect is not likely to be due to the bicarbonate contents of the fluids.

Hamner and Williams (1963, 1964) and Murdoch and White (1968) are of the opinion that bicarbonate in oviduct fluid from the rabbit stimulates the respiration and glycolysis of spermatozoa. Foley and Williams (1967), while finding an increase in respiration due to bicarbonate, conclude that other stimulants to spermatozoal respiration are present in the tubal secretions of the sow, ewe, and rabbit. Reports from this laboratory (Restall and Wales 1966; Wales and Restall 1966) have not demonstrated a stimulus to the respiration of ram spermatozoa in the presence of oviduct fluid. Thus it seems likely that a species difference is present. Further evidence of differences between species is furnished by the fact that Murdoch and White (1968) found no Pasteur effect in rabbit spermatozoa, while ram spermatozoa exhibited a distinct Pasteur effect (see experiment 4) which was abolished by tubal fluid.

Although both Tris and veronal buffers may affect cellular function (Quastel and Wheatley 1933; Omachi, Macey, and Waldeck 1961), they have been found suitable for use in semen diluents (Blackshaw 1953; Wales and White 1958). In the present experiments, the choice of buffer influenced the magnitude of both the respiratory and glycolytic response of the spermatozoa. In particular, bicarbonate buffer appeared to enhance glycolysis in the saline controls and depressed it when uterine and tubal fluids were present. This had the effect of greatly reducing the magnitude of the stimulation to glycolysis afforded by tubal fluid, when compared with spermatozoa incubated in phosphate buffer.

The relative greater sensitivity of the saline controls to changes in incubation conditions is also illustrated by the amount of lactate formed from the glucose utilized. In saline controls the ratio of lactate formed to the glucose utilized varies between 1·5:1 and 2:1 depending on the buffer used. With flasks containing tubal fluid there is quantitative conversion of glucose to lactate irrespective of the buffer used. Thus tubal fluid appears to have a stabilizing effect on the incubation medium such that changes in conditions do not have as pronounced effects as are observed in the saline controls.

The experiments reported here demonstrate that modifications to the experimental conditions have significant effects on the reaction of ram spermatozoa to the presence of tubal fluid. However, under a variety of experimental conditions, a depression of respiration and a stimulation of glycolysis consistently occurred whenever ram spermatozoa were incubated in significant amounts of tubal fluid. At the same time, dilution of fluid or changes in the conditions of incubation modified the magnitude of these effects.

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VI. References

Barker, J. N., and Britton, H. G. (1957).—The enzymatic estimation of L-(+) lactic acid. *J. Physiol.* **138**, 3P.

Blackshaw, A. H. (1953).—The effects of potassium and calcium salts on the motility of ram, rabbit and bull spermatozoa. *J. Physiol.* **120**, 465.

Foley, C. S., and Williams, W. L. (1967).—Effect of bicarbonate and oviduct fluid on respiration of spermatozoa. *Proc. Soc. exp. Biol. Med.* **126**, 634–7.

Hamner, C. E., and Williams, W. L. (1963).—Effects of the female reproductive tract on sperm metabolism in the rabbit and fowl. *J. Reprod. Fert.* **5**, 143–50.

Hamner, C. E., and Williams, W. L. (1964).—Identification of sperm stimulating factor of rabbit oviduct fluid. *Proc. Soc. exp. Biol. Med.* **117**, 240–3.

Huggett, A. St. G., and Nixon, D. A. (1957).—Enzymic determination of blood glucose. *Biochem. J.* **66**, 12P.

Kirton, K. T., and Hafs, H. D. (1965).—Sperm capacitation by uterine fluid or beta-amylase *in vitro*. *Science, N.Y.* **150**, 618–19.

Mann, T. (1948).—Fructose content and fructolysis in semen. Practical application in the evaluation of semen quality. *J. agric. Sci.* **38**, 232.

Murdoch, R. N., and White, I. G. (1968).—The influence of the female genital tract on the metabolism of rabbit spermatozoa. I. Direct effect of tubal and uterine fluids, bicarbonate, and other factors. *Aust. J. biol. Sci.* **12**, 961–72.

Olds, D., and Vandemark, N. L. (1957).—The behaviour of spermatozoa in luminal fluids of bovine female genitalia. *Am. J. vet. Res.* **18**, 603–7.

Omachi, A., Macey, R. I., and Waldeck, J. G. (1961).—Permeability of cell membranes to amine buffers and their effects on electrolyte transport. *Ann. N.Y. Acad. Sci.* **92**, 478.

O'Shea, T., and Wales, R. G. (1964).—Effects of potassium on ram spermatozoa during chilling to and storage at 5°C. *J. Reprod. Fert.* **11**, 171–80.

O'Shea, T., and Wales, R. G. (1965).—Metabolism of sorbitol and fructose by ram spermatozoa. *J. Reprod. Fert.* **10**, 353–68.

QUASTEL, J. H., and WHEATLEY, A. H. M. (1933).—Narcosis and oxidations of the brain. *Proc. R. Soc.* B **112**, 60.

RESTALL, B. J. (1966).—The fallopian tube of the sheep. I. Cannulation of the fallopian tube. *Aust. J. biol. Sci.* **19**, 181–6.

RESTALL, B. J., and WALES, R. G. (1966).—The fallopian tube of the sheep. IV. The metabolism of ram spermatozoa in the presence of fluid from the fallopian tube. *Aust. J. biol. Sci.* **19**, 883.

WALES, R. G., and O'SHEA, T. (1966).—Effect of low levels of carbon dioxide on the metabolism of ram and bull spermatozoa. *J. Reprod. Fert.* **11**, 171–80.

WALES, R. G., and RESTALL, B. J. (1966).—The metabolism of ram spermatozoa in the presence of genital fluids of the ewe. *Aust. J. biol. Sci.* **19**, 199–209.

WALES, R. G., and WHITE, I. G. (1958).—The interaction of pH, tonicity, and electrolyte concentration on the motility of dog spermatozoa. *J. Physiol.* **141**, 273.

WHITE, I. G. (1959).—Studies on the estimation of glycerol, fructose and lactic acid with particular reference to semen. *Aust. J. exp. Biol. med. Sci.* **37**, 441.