METABOLIC STUDIES OF WASHED AND DILUTED RAM AND BULL SPERMATOZOA

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Summary

The total oxygen uptake of ram and bull spermatozoa in a 3-hr period at 37°C was not affected by washing four times in a sodium phosphate-fructose diluent nor by centrifuging four times without washing.

The total lactic acid production of the spermatozoa of both species was significantly reduced by washing four times but was unaffected by merely centrifuging. Washed ram and bull spermatozoa produced more lactic acid when 0.004M KCl was added to the sodium phosphate-fructose diluent; the glycolysis of ram spermatozoa washed in the potassium-containing diluent was almost equal to the unwashed control.

The total oxygen uptake of ram and bull spermatozoa was not affected by a tenfold dilution from an initial cell concentration of 200×10^6 cells/ml when the dilution was made in either a potassium-containing or a potassium-free diluent. Dilution significantly reduced the lactic acid production of the spermatozoa of both species, particularly when the dilution was made in a potassium-free diluent. The addition of 0.004M KCl to the sodium phosphate-fructose diluent increased the glycolysis of diluted ram spermatozoa but not that of diluted bull spermatozoa.

It is concluded from these experiments that repetitive washing and dilution are similar in their effect on the metabolism of ram and bull spermatozoa and that it is the glycolytic rather than the oxidative mechanisms that are damaged. Potassium appears to be one important component lost from spermatozoa during both treatments.

I. INTRODUCTION

In previous studies (White 1953*a*) it was found that ram, bull, and rabbit spermatozoa could be washed once in a sodium phosphate-fructose diluent with little or no impairment of motility or metabolism. On washing twice there was a decrease in motility, total oxygen uptake, and total lactic acid production over the 5-hr period of the experiments. While the effect of repeated washing on the total oxygen consumption was shown to be largely due to the removal of bacteria otherwise proliferating in the suspensions during the later hours, it was believed that the decreased lactic acid production was associated with the spermatozoa. To examine these conclusions more critically the effect of washing on the oxygen uptake and glycolysis of ram and bull spermatozoa has been investigated, over a 3-hr period to minimize the influence of bacteria, with the number of washings increased to four to accentuate this effect.

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Motility studies of ram and bull spermatozoa after washing four times have already been reported (White 1953*b*). In these experiments it was shown that the adverse effect of repeated washing on motility could be largely prevented by the inclusion of potassium in the diluent, and furthermore, that potassium had a beneficial action on spermatozoa at moderate dilutions. Similar results have been obtained by Blackshaw (1953*a*, 1953*b*). A survey of the available literature revealed only one reference to the effect of potassium on the metabolism of mammalian spermatozoa (Lardy and Phillips 1943), although numerous studies have been made of other cells and tissues, mostly in relation to the importance of potassium in carbohydrate metabolism. Thus it has been shown that potassium stimulates the aerobic metabolism of brain (Ashford and Dixon 1935; Lipsett and Crescitelli 1950), fermentation by yeast (Farmer and Jones 1942), and glycogen deposition in rat liver slices (Buchanan, Hastings, and Nesbett 1949*a*, 1949*b*). It was of interest therefore to study the effect of potassium on the metabolism of washed and diluted spermatozoa, and the results of these experiments are reported here.

II. Materials and Methods

Bull semen was collected by the artificial vagina and ram semen by electrical stimulation (Gunn 1936). Only morphologically normal ejaculates of good motility were employed. Experiments with ram semen were started immediately after collection and those with bull semen within 2 hr. The semen was stored during this period at about 10°C, precautions being taken to avoid cold shock.

Suspensions were prepared by diluting 0.5-1.0 ml of semen 1 : 10 in a graduated centrifuge tube, and the spermatozoa were washed by spinning this suspension four times at 1500 r.p.m. (about 300g) for 10 min. The supernatant that was drawn off and replaced by diluent after each centrifuging and the spermatozoa redispersed. Centrifuged but unwashed spermatozoa suspensions were prepared by centrifuging the diluted semen four times and redispersing the cells after each spinning without removing the supernatant.

In the dilution experiments spermatozoal counts were made with the neat semen, using a haemocytometer under high power. The semen was then serially diluted to give cell concentrations of *c.* 200×10^6 and 20×10^6 cells/ml.

Each experiment was of 3 hr duration, the gas phase being air. The Warburg bath was set at 37°C with a shaking rate (114 strokes/min) sufficient to keep the liquid phase saturated with oxygen. Oxygen uptake was measured at hourly intervals by the direct Warburg technique (Umbreit, Burris, and Stauffer 1949).

Lactic acid was estimated on the flask contents by the method of Barker and Summerson (1941) at the start and end of experiments.

The isotonic diluents of pH 7.0 had the following composition:

Sodium: 0.032M $NaH_2PO_4.H_2O$, 0.048M $Na_2HPO_4.12H_2O$, 0.040M NaCl, 0.022M fructose.

Sodium-potassium: 0.032M $NaH_2PO_4.H_2O$, 0.048M $Na_2HPO_4.12H_2O$, 0.036M NaCl, 0.004M KCl, 0.022M fructose.

In the analysis of variance the total oxygen uptake and the total lactic acid production over the 3-hr period have been used as unit observations and the interaction mean square as the error term. The treatment sum of squares has been partitioned to give selected comparisons which are non-orthogonal.

TABLE 1

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE OXYGEN UPTAKE OF RAM SPERMATOZOA AT 37°C

Treatment	Ejaculate	$O_2/10^8$ Cells/Hr (μl)			Total Oxygen Uptake
		First Hr	Second Hr	Third Hr	
Unwashed	1	9·7	5·9	5·5	21·1
	2	12·7	8·4	8·6	29·7
	3	11·2	7·0	6·6	24·8
	Mean	11·2	7·1	6·9	25·2
Centrifuged but unwashed	1	8·7	5·7	6·9	21·3
	2	10·2	6·9	7·3	24·4
	3	10·6	5·5	5·8	21·9
	Mean	9·8	6·0	6·7	22·5
Washed	1	10·7	7·4	5·4	23·5
	2	11·2	9·7	5·6	26·5
	3	11·0	7·1	6·2	24·3
	Mean	11·0	8·1	5·7	24·7

III. RESULTS

The effect of washing four times on the oxygen uptake and glycolysis of ram and bull spermatozoa over a 3-hr period was studied with three ejaculates from each species, using the sodium phosphate-fructose diluent. A centrifuged but unwashed group was included in these experiments in addition to the unwashed control, to assess the mechanical effect of centrifuging on the oxygen uptake of the spermatozoa. Tables 1 and 2 give the results for the oxygen uptake of ram and bull spermatozoa respectively. These data were subjected to analysis of variance and it is clear from the summary (Table 3) that there was no significant difference between either treatments or ejaculates in the two species.

The corresponding values for lactic acid production are set out in Table 4. These also were subjected to analysis of variance (Table 5) which showed that washing four times significantly reduced the amount of lactic acid produced by ram and bull spermatozoa. Lactic acid production by the centrifuged but unwashed spermatozoa of both species was not, on the other hand, significantly different from that in the control specimens that had been neither centrifuged nor washed. The ejaculates from the bulls used in this experiment varied

significantly in their capacity to produce lactic acid; this was not so, however, with the ram ejaculates.

TABLE 2

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE OXYGEN UPTAKE OF BULL SPERMATOZOA AT 37°C

Treatment	Ejaculate	$O_2/10^8$ Cells/Hr (μl)			Total Oxygen Uptake
		First Hr	Second Hr	Third Hr	
Unwashed	1	6·5	5·0	5·5	17·0
	2	8·0	5·5	5·1	18·6
	3	—	—	—	32·0*
	Mean				22·5
Centrifuged but unwashed	1	7·3	5·8	6·0	25·1
	2	8·1	7·7	4·5	20·3
	3	14·5	12·1	9·2	35·8
	Mean	10·0	8·5	6·6	25·1
Washed	1	5·6	5·8	6·5	17·9
	2	8·3	10·2	3·7	22·2
	3	15·2	9·9	7·2	32·3
	Mean	9·7	8·6	5·8	24·1

* This value has been calculated by the method described in Cochran and Cox (1950) for estimating missing data.

TABLE 3

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE TOTAL OXYGEN UPTAKES IN TABLES 1 AND 2

Source of Variation	Ram		Bull	
	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
Between treatments:				
Unwashed *v.* washed	1	0·1	1	1·5
Unwashed *v.* centrifuged	1	4·1	1	3·7
Between ejaculates	2	7·2	2	7·9
Interaction (error)	4	258	3*	259

* The degrees of freedom have been reduced by one because of the missing value inserted in Table 2

It has been shown previously (White 1953*b*) that the addition of potassium to the sodium diluent almost completely restores the motility of repeatedly washed ram spermatozoa, and markedly increases that of washed bull spermatozoa. Experiments were therefore undertaken to see if potassium had a similar

effect on the depressed glycolysis of the washed spermatozoa. Five ram and five bull ejaculates were used. With each ejaculate comparisons were made between spermatozoa washed four times in the potassium-containing and potassium-free diluents respectively, with an unwashed specimen in the potassium-free diluent as a control. The results are given in Table 6 and the summary of the analyses of variance in Table 7. It is clear that lactic acid production by the washed spermatozoa of both species is significantly greater in the presence of 0.004M KCl. The addition of potassium to the washed ram spermatozoa almost restored glycolysis to the level of the unwashed controls.

TABLE 4

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE GLYCOLYSIS OF RAM AND BULL SPERMATOZOA AT 37°C

Values represent μg lactic acid produced by 10^8 spermatozoa in 3 hr

Ejaculate	Unwashed		Centrifuged but Unwashed		Washed	
	Ram	Bull	Ram	Bull	Ram	Bull
1	114	168	106	196	8	101
2	137	326	147	382	−3	173
3	82	182	117	153	−22	79
Mean	111	225	123	244	−6	118

TABLE 5

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 4

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
Unwashed *v.* washed	1	37·6**	11·6*
Unwashed *v.* centrifuged	1	0·4	0·3
Between ejaculates	2	1·7	14·6*
Interaction (error)	4	542	1503

* $P<0·05$.
** $P<0·01$.

A feature of mammalian spermatozoa is that their motility is decreased by dilution (Salisbury *et al.* 1943; Emmens and Swyer 1948; Cheng, Casida, and Barrett 1949; White 1953*b*; Blackshaw 1953*b*) even if the dilution is made in an isotonic buffered medium containing a glycolysable sugar. The damage caused by dilution might be expected to be similar to that produced by repeated washing, since in both procedures the concentration of seminal plasma consti-

tuents is reduced and the loss of intracellular material from the spermatozoa presumably accelerated thereby.

TABLE 6

EFFECTS OF WASHING FOUR TIMES IN SODIUM AND SODIUM-POTASSIUM DILUENTS ON THE GLYCOLYSIS OF RAM AND BULL SPERMATOZOA AT 37°C

Values represent μg lactic acid produced by 10^8 spermatozoa in 3 hr

Ejaculate	*A.* Unwashed in Sodium Diluent		*B.* Washed in Sodium Diluent		*C.* Washed in Sodium-Potassium Diluent	
	Ram	Bull	Ram	Bull	Ram	Bull
1	246	277	45	223	164	275
2	275	146	222	29	411	44
3	221	363	97	88	211	219
4	330	290	131	153	289	260
5	433	411	163	116	345	245
Mean	301	297	132	122	284	209

TABLE 7

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 6

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	26·2**	24·8**
B v. C	1	21·2**	6·1*
Between ejaculates	4	5·8*	5·8*
Interaction (error)	8	2739	3115

* $P<0·05$.
** $P<0·01$.

It was of interest therefore to see if dilution had a similar effect to repeated washing on the metabolism of spermatozoa and to investigate the influence of potassium. Four ejaculates from each species were used. Table 8 gives the lactic acid production and Table 10 the oxygen uptake of ram and bull spermatozoa incubated for 3 hr at 37°C after 1 : 10 dilution in sodium and sodium-potassium diluents, the cells being suspended initially in the sodium diluent at a concentration of 200×10^6 ml. Ideally, it would have been desirable to work at higher dilutions since previous motility studies have shown that the dilution phenomenon is greatest at very low cell concentrations; the degree of dilution

possible in metabolic experiments is, however, restricted by the design of the Warburg apparatus and the sensitivity of the techniques used. The analyses of variance for the respiration data are summarized in Table 11 and show that the oxygen uptake of ram and bull spermatozoa was not significantly affected by dilution in either the sodium or sodium-potassium diluents. On

TABLE 8

LACTIC ACID (μg) PRODUCED BY 10^8 RAM AND BULL SPERMATOZOA INCUBATED FOR 3 HR AT 37°C AFTER DILUTION IN SODIUM AND SODIUM-POTASSIUM DILUENTS

Ejaculate	*A.* Sodium Diluent (200×10^6 cells/ml)		*B.* Sodium Diluent (20×10^6 cells/ml)		*C.* Sodium-Potassium Diluent (20×10^6 cells/ml)	
	Ram	Bull	Ram	Bull	Ram	Bull
1	240	435	94	206	177	202
2	549	250	178	62	260	125
3	505	208	154	54	325	208
4	267	675	35	276	214	275
Mean	390	392	115	150	269	203

TABLE 9

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 8

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	48·4**	15·3**
B v. C	1	15·1**	0·7
Between ejaculates	3	9·9	6·0*
Interaction (error)	6	3128	7679

* $P<0·05$.
** $P<0·01$.

the other hand, analysis of variance of the glycolysis results (Table 9) shows that dilution in the sodium diluent significantly reduced the lactic acid production of the spermatozoa of both species. The glycolysis of ram spermatozoa on dilution was significantly greater in the presence of potassium, but this effect was not seen with bull spermatozoa. Significant between-ejaculate variation in oxygen consumption (Table 11) occurred with the rams used in this experiment, whilst the lactic acid production of different ejaculates differed significantly in both species (Table 9).

IV. Discussion

In these experiments it is shown that washing ram and bull spermatozoa four times in a sodium phosphate-fructose diluent causes a fall in their lactic acid production (Table 4). These results confirm those previously reported by White (1953*a*) in which it was demonstrated that even after two washings the lactic acid production of ram, bull, and rabbit spermatozoa was significantly impaired. The effect was much more striking after the exhaustive washing employed in the experiments described here and, as they were conducted over a shorter period, the results are less likely to be complicated by the proliferation of bacteria. On the other hand, the total oxygen uptake of ram and bull spermatozoa (Tables 1 and 2) in these experiments was not affected by repetitive washing.

Table 10

OXYGEN (μl) TAKEN UP BY 10^8 RAM AND BULL SPERMATOZOA INCUBATED FOR 3 HR AT 37°C AFTER DILUTION IN SODIUM AND SODIUM-POTASSIUM DILUENTS

Ejaculate	*A.* Sodium Diluent (200×10^6 cells/ml)		*B.* Sodium Diluent (20×10^6 cells/ml)		*C.* Sodium-Potassium Diluent (20×10^6 cells/ml)	
	Ram	Bull	Ram	Bull	Ram	Bull
1	43·2	24·3	49·0	31·4	50·0	37·4
2	27·7	33·2	26·9	27·4	40·5	36·4
3	21·3	21·5	41·3	30·8	35·0	23·1
Mean	33·3	28·6	41·7	26·6	41·5	30·3

Table 11

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 10

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	3·8	0·5
B v. C	1	3·6	3·2
Between ejaculates	3	5·0*	4·4
Interaction (error)	6	3763	2273

* $P<0·05$.

The adverse effect of repeated washing would seem therefore to involve damage to the glycolytic rather than to the oxidative processes of the spermatozoa. Furthermore, the decreased glycolysis must be associated with the leaching out of intracellular components from the spermatozoa rather than with

the mechanical damage due to centrifuging, since the spermatozoa that were centrifuged without washing produced as much lactic acid as the unwashed controls (Table 4).

The motility observations of White (1953*b*) and Blackshaw (1953*b*) suggested that potassium was an important substance lost from spermatozoa on repeated washing, and the metabolic studies reported here support this suggestion.

The effect of dilution on the metabolism of ram and bull spermatozoa (Tables 8 and 10) was similar to that of repetitive washing. Potassium loss from the spermatozoa cannot be the only factor involved, however, since the lactic acid production of ram spermatozoa diluted in the potassium medium was never as high as that of the undiluted controls, whilst potassium had no significant effect on the glycolysis of diluted bull spermatozoa (Table 9).

The observation that the respiratory rate of ram and bull spermatozoa was unaffected by a tenfold dilution from an initial cell concentration of 200×10^6/ml is supported by several workers (Lardy and Phillips 1941; Lardy, Winchester, and Phillips 1945; Salisbury 1946). At much higher cell concentrations Winchester and McKenzie (1941) working with ram, and Salisbury (1946) with bull spermatozoa, record a depression of oxygen uptake. As has been pointed out, however, by Lardy, Winchester, and Phillips (1945) this might well be the result of limited oxygen diffusion into the viscous liquid phase.

Mann and Lutwak-Mann (1948) found a similar fall (Table 8) in the lactic acid production of ram spermatozoa after a fivefold dilution from an initial concentration of 3×10^8 cells/ml. However, these workers also recorded a reduction of oxygen uptake on dilution. Whilst this might be so with some ejaculates, the results of the experiments reported here indicate that impairment of glycolysis precedes that of oxidative processes. Salisbury (1946) seems to be the only other worker who has studied the effect of dilution on the glycolysis of mammalian spermatozoa and his results appear to be at variance with those reported here, in that he was unable to detect any difference in the glycolysis of bull spermatozoa at dilutions of 1 : 4 and 1 : 16. His experiments were, however, conducted over a 10-day period and the results might easily have been complicated by bacterial growth. Furthermore, over this long period, the metabolism of the spermatozoa at the higher concentration may have been depressed by a fall in the pH of the diluent.

Apparently the only reference in the literature to the effect of potassium on the metabolism of mammalian spermatozoa is that of Lardy and Phillips (1943), who present evidence for the stimulatory action of potassium on both the respiration and glycolysis of bull spermatozoa washed twice in potassium-free Ringer phosphate. Whilst neither effect could be judged statistically significant, it is clear from their limited data that the effect of potassium on glycolysis is much greater than on respiration.

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VI. References

Ashford, C. A., and Dixon, K. D. (1935).—The effect of potassium on the glycolysis of brain tissue with reference to the Pasteur effect. *Biochem. J.* **29**: 157-63.

Barker, S. B., and Summerson, W. H. (1941).—The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* **138**: 535-54.

Blackshaw, A. W. (1953*a*).—The effect of potassium and calcium salts on the motility of ram, bull and rabbit spermatozoa. *J. Physiol.* **20**: 465-70.

Blackshaw, A. W. (1953*b*).—The motility of ram and bull spermatozoa in dilute suspension. *J. Gen. Physiol.* **36**: 449-62.

Buchanan, J. M., Hastings, A. B., and Nesbett, F. B. (1949*a*).—The effect of the ionic environment on the synthesis of glycogen from glucose in rat liver slices. *J. Biol. Chem.* **180**: 435-45.

Buchanan, J. M., Hastings, A. B., and Nesbett, F. B. (1949*b*).—The effect of ionic environment on the synthesis of glycogen and total carbohydrate from pyruvate in liver slices. *J. Biol. Chem.* **180**: 447-55.

Cheng, P., Casida, L. E., and Barrett, G. R. (1949).—Effects of dilution on motility of bull spermatozoa and the relation between motility in high dilution and fertility. *J. Anim. Sci.* **8**: 81-8.

Cochran, W. G., and Cox, G. M. (1950).—"Experimental Designs." (John Wiley & Sons: New York.)

Emmens, C. W., and Swyer, G. I. M. (1948).—Observations on the motility of rabbit spermatozoa in dilute suspension. *J. Gen. Physiol.* **32**: 121-38.

Farmer, S. N., and Jones, D. A. (1942).—Influence of potassium on sugar metabolism. *Nature* **150**: 768-9.

Gunn, R. M. C. (1936).—Fertility in sheep. Coun. Sci. Industr. Res. Aust. Bull. No. 94.

Lardy, H. A., and Phillips, P. H. (1941).—Phospholipids as a source of energy for motility of bull spermatozoa. *Amer. J. Physiol.* **134**: 542-8.

Lardy, H. A., and Phillips, P. H. (1943).—Effect of pH and certain electrolytes on the metabolism of ejaculated spermatozoa. *Amer. J. Physiol.* **138**: 741-6.

Lardy, H. A., Winchester, B., and Phillips, P. H. (1945).—The respiratory metabolism of ram spermatozoa. *Arch. Biochem.* **6**: 33-40.

Lipsett, M. N., and Crescitelli, F. (1950).—The effects of increased potassium concentration on the metabolism of rat cerebral cortical slices. *Arch. Biochem.* **28**: 329-37.

Mann, T., and Lutwak-Mann, C. (1948).—Aerobic and anaerobic utilization of fructose by spermatozoal and seminal vesicles. *Biochem. J.* **43**: 266-70.

Salisbury, G. W. (1946).—"The Problem of Fertility." (Princeton University: Princeton, N.J.)

Salisbury, G. W., Beck, G. H., Cupps, P. T., and Elliot, I. (1943).—The effect of dilution rate on the livability and the fertility of bull spermatozoa used for artificial insemination. *J. Dairy Sci.* **26**: 1057-69.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1949).—"Manometric Techniques and Tissue Metabolism." (Burgess: Minneapolis.)

White, I. G. (1953*a*).—The effect of washing on the motility and metabolism of ram, bull and rabbit spermatozoa. *J. Exp. Biol.* **30**: 200-13.

White, I. G. (1953*b*).—The effect of potassium on the washing and dilution of mammalian spermatozoa. *Aust. J. Exp. Biol. Med. Sci.* **31**: 193-200.

Winchester, C. F., and McKenzie, F. F. (1941).—Influence of H-ion concentration on respiration of ram sperm. *Proc. Soc. Exp. Biol., N.Y.* **48**: 654-6.