THE EFFECT OF DILUTION ON THE CONCENTRATION OF SODIUM, POTASSIUM, CALCIUM AND MAGNESIUM IN RAM AND BULL SPERMATOZOA

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Summary. Mild dilution (<1.8) of ram and bull semen in isotonic sodium phosphate diluent of pH 7.0 caused an influx of sodium and an efflux of potassium, calcium and magnesium from the spermatozoa. Equilibrium was reached soon after dilution and little further change in the concentration of these cations was observed over 3 hr.

Similar changes occurred when ram and bull semen was diluted in tris and veronal diluents of pH 7; however, calcium was found to accumulate in ram spermatozoa diluted in tris. Diluting semen in buffers of pH range 8.5 to 5.5 indicated that potassium loss from the spermatozoa increased with increasing pH.

Greater dilution (1:100) and exhaustive washing (four times) had a similar but more severe effect on the cation concentration of ram and bull spermatozoa. A large influx of sodium was accompanied by a marked depletion of potassium which tended to be further depleted over 3 hr.

INTRODUCTION

When mammalian semen is excessively diluted or washed, even in an isotonic medium containing a glycolysable sugar, the viability of the spermatozoa is impaired (Milovanov, 1934; Chang, 1947, 1949; Emmens & Swyer, 1948; Blackshaw, 1953a, b; White, 1953c, d). This is probably due to the removal of essential components from inside the cell (cf. also White, 1953a, b). A number of other authors have also demonstrated the beneficial effect of potassium on the viability of washed or diluted spermatozoa (Wales & White, 1958a, b, 1961, 1963; Dott & White, 1964; Wallace & Wales, 1964) and it has been suggested that potassium may be one of the important substances lost from spermatozoa.

The potassium concentration is greater in spermatozoa than in the seminal plasma (Cragle, Salisbury & Muntz, 1958; Cragle, Salisbury & VanDemark, 1958; Quinn, White & Wirrick, 1965) and potassium is lost from ram spermatozoa even when the semen is subjected to very mild dilutions in a sodium phosphate buffer (Dott & White, 1964). This paper is concerned with further

studies on the effect of dilution on the potassium, sodium, calcium and magnesium content of ram and bull spermatozoa.

MATERIALS AND METHODS

Ram semen was collected by electrical ejaculation using the method of Blackshaw (1954) and bull semen using an artificial vagina. Cold shocking of the semen was avoided and only samples of good initial motility were used.

Aliquots (0.5 to 1.0 ml) of semen—either neat or diluted up to 1:8—were centrifuged in special tapering centrifuge tubes to separate the spermatozoa (Quinn *et al.*, 1965). The 1:100 dilution was made in large centrifuge tubes and the spermatozoa spun down to about 5 ml before transferring to the special tubes for further centrifuging. Spermatozoa were washed by diluting 0.5 ml of semen to 4.5 ml and centrifuging at 1700 rev/min for 10 min and withdrawing the supernatant (White, 1953a); this procedure was repeated four times.

An atomic absorption spectrophotometer was used for the analysis of sodium, potassium, calcium and magnesium (Quinn *et al.*, 1965).

Dott & White's (1964) 0.08 M-phosphate diluent was used for diluting ram semen. This concentration of phosphate was found to depress the motility of bull spermatozoa and 0.02 M-phosphate was therefore used routinely for this species. The 0.08 M-sodium phosphate diluents consisted of 0.048 M- $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.032 M- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.040 M-NaCl, (pH 7), and 0.0008 M- $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.079 M- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.072 M-NaCl, (pH 5.5). The 0.02 M-phosphate diluents contained a quarter of the concentration of the mono- and di-sodium phosphate and 0.120 M and 0.144 M-NaCl for pH 7 and 5.5 respectively.

Tris-HCl diluent consisted of 0.042 M-tris [*tris*-(hydroxy-methyl)-amino-methane]; 0.038 M-HCl, pH 7), and 0.062 M-tris; 0.019 M-HCl, (pH 8.5). 0.104 M-NaCl was added to both. Veronal-HCl diluent of pH 7 consisted of 0.0033 M-sodium diethyl-barbiturate; 0.030 M-HCl and 0.080 M-NaCl. Fructose (400 mg/100 ml) was added to all diluents as a substrate for the spermatozoa and the final mixtures were isotonic with 0.9% NaCl.

The statistical significance of the results has been assessed by an analysis of variance, often using the SILLIAC electronic computer. The interaction mean square has been used to calculate the standard errors (S.E.) of the differences between means as presented in the tables, and this has been used for the *t*-test.

RESULTS

Effect of dilution in phosphate buffer

Ram and bull semen were diluted two-, four- and eight-fold in sodium phosphate buffer of pH 7 and the spermatozoa immediately separated from the supernatant. The concentrations of sodium, potassium, calcium and magnesium in the spermatozoa at each dilution are presented in Table 1 (Experiment 1).

Sodium accumulates in the spermatozoa on dilution and potassium, calcium and magnesium are lost, the effects being more marked with increasing dilution. The exchanges of sodium, potassium and magnesium are similar for ram and bull spermatozoa. However, the calcium content of undiluted bull spermatozoa (29.6 mg/100 g) is higher than ram (10.1 mg/100 g), but they become similar

(7.1 and 10.4 mg/100 g respectively) on 1:8 dilution. The loss of calcium is thus much greater with bull spermatozoa.

The influx of sodium at a dilution of 1:8 (29 and 50 m-equiv/l for ram and bull respectively) is almost twice the combined loss of potassium, calcium and magnesium (15 and 28 m-equiv/l for ram and bull respectively).

Effect of time on dilution changes

Ram and bull semen were diluted 1:2 in sodium phosphate diluents of pH 7 and cation analyses were made on the spermatozoa initially and at hourly intervals for 3 hr at room temperature (15 to 20° C). The results for the diluted and neat semen samples are summarized in Table 1 (Experiment 2).

TABLE 1
CONCENTRATION (mg/100 g) OF CATIONS IN SPERMATOZOA AFTER DILUTING SEMEN IN SODIUM PHOSPHATE BUFFERS (pH 7.0)

Experiment	Species	Treatment	Sodium	Potassium	Calcium	Magnesium
1	Ram (seven replicates)	Undiluted 0 hr	109	141	10.1	15.1
		1:2 dilution 0 hr	167**	118	7.2	12.2
		1:4 dilution 0 hr	180**	109*	8.1	12.5
		1:8 dilution 0 hr	175**	96**	7.1	10.4*
			S.E. = 20	S.E. = 12	S.E. = 2.4	S.E. = 1.7
	Bull (four replicates)	Undiluted 0 hr	122	159	29.6	15.6
		1:2 dilution 0 hr	183*	133	17.7*	11.4*
		1:4 dilution 0 hr	170	100**	9.9**	9.9**
		1:8 dilution 0 hr	234**	92**	10.4**	8.5**
			S.E. = 26	S.E. = 15	S.E. = 5.3	S.E. = 1.3
2	Ram (nine replicates)	Undiluted 0 hr	116**	135**	8.1**	13.7
		1:2 dilution 0 hr	192	95	5.4	12.8
		1 hr	159*	89	5.5	12.1
		2 hr	174	99	7.2	14.1
		3 hr	169	81	7.5*	14.3
			S.E. = 14	S.E. = 7	S.E. = 0.8	S.E. = 1.6
	Bull (six replicates)	Undiluted 0 hr	125*	227**	25.2**	13.3
		1:2 dilution 0 hr	202	138	15.2	11.6
		1 hr	201	145	11.7	11.5
		2 hr	235	148	9.0*	9.9
		3 hr	189	131	8.9*	9.5
			S.E. = 34	S.E. = 24	S.E. = 3.0	S.E. = 1.5

* $P < 0.05$; ** $P < 0.01$ (probability of treatment being different from undiluted in Experiment 1 and diluted 0 hr in Experiment 2).

As in the previous experiment there was a rise in sodium and a fall in potassium, calcium and magnesium, but the effect was usually immediate and there was little further change during the 3-hr incubation.

Effect of dilution in different buffers

Ram and bull semen were diluted 1:8 and 1:4 respectively in phosphate, tris and veronal buffers of pH 7 and the concentrations of sodium, potassium, calcium and magnesium were determined.

Table 2 (Experiment 3) shows that tris and veronal buffers, like phosphate,

TABLE 2
THE EFFECT OF DIFFERENT BUFFERS AND pH ON THE CONCENTRATION (mg/100 g) OF CATIONS IN SPERMATIZOEA

Experiment	Species	Dilution	Buffer	pH	Sodium	Potassium	Calcium	Magnesium
3	Ram (six replicates)	Undiluted 1:8	-	-	122	131	5.9	14.2
			0.08 M-phosphate	7.0	174**	96**	5.2	14.1
			tris	7.0	134	98**	12.0**	15.4
			veronal	7.0	115	86**	6.5	12.4
					S.E. = 12	S.E. = 6	S.E. = 1.5	S.E. = 0.9
	Bull (four replicates)	Undiluted 1:4	-	-	132	192	25.0	14.9
			0.08 M-phosphate	7.0	253*	135*	11.1**	12.9*
			0.02 M-phosphate	7.0	279**	148*	12.0**	12.9**
			tris	7.0	267**	127**	12.5**	11.5**
			veronal	7.0	241*	116**	11.6**	10.2**
					S.E. = 43	S.E. = 19	S.E. = 3.3	S.E. = 0.8
4	Ram (six replicates)	Undiluted 1:8	-	-	126**	163**	17.6	17.8
			0.08 M-phosphate	5.5	232*	105	15.0	20.1
			tris	7.0	283	106	17.8	18.1
			8.5	193**	112	28.4**	20.3	20.3
					228*	71*	17.4	18.4
					S.E. = 24	S.E. = 15	S.E. = 3.0	S.E. = 2.1
	Bull (six replicates)	Undiluted 1:4	-	-	162	214**	25.3**	15.8*
			0.02 M-phosphate	5.5	261	127	12.7	14.4
			tris	7.0	233	97	14.9	11.7
			8.5	291	139	10.8	11.4	11.4
					248	100	12.7	13.4
					S.E. = 64	S.E. = 28	S.E. = 3.0	S.E. = 2.4

* $P < 0.05$; ** $P < 0.01$ (probability of treatment being different from undiluted in Experiment 3 and phosphate pH 7.0 in Experiment 4).

all caused a significant increase in the sodium concentration of bull spermatozoa and a decrease in potassium, calcium and magnesium. The rise in the sodium concentration of ram spermatozoa was less with tris and veronal, although all three buffers gave a comparable depression of potassium and had no significant effect on magnesium. Diluting ram spermatozoa in tris caused, surprisingly, an increase in the calcium content.

Diluting ram and bull semen in the veronal diluent and bull semen in the 0.08 M-phosphate diluent considerably depressed the motility of the spermatozoa.

Effect of pH on diluted spermatozoa

The effect of the pH of the diluent on the concentration of the major cations in diluted ram and bull spermatozoa is shown in Table 2 (Experiment 4). Two buffers were used, phosphate and tris, to give pH values of 5.5, 7.0 and 8.5; both buffers were used at the overlapping pH of 7.0 to control any differences due to the buffer rather than to pH.

The most consistent effect is that potassium loss tends to increase with increasing pH. Thus potassium values are lower at 8.5 than at 7.0 for ram and bull spermatozoa diluted in tris and lower at 7.0 and 5.5 for bull spermatozoa diluted in phosphate.

There is also evidence of a reverse effect of pH on the sodium concentration of ram spermatozoa; thus the sodium values are higher at 8.5 than at 7.0 for tris and higher at 7.0 than 5.5 in phosphate. As in the previous experiment, sodium influx is less for ram spermatozoa diluted in tris than in phosphate at pH 7.0. Again there was an accumulation of calcium when ram spermatozoa was diluted in tris at pH 7.0. However, at pH 8.5 there was no accumulation of this cation.

Effect of high dilution and severe washing

The cation concentration of ram and bull spermatozoa after high dilution (1:100) and after washing four times in a phosphate buffer is shown in Table 3. Analyses were made directly after diluting or washing and again after incubation for 3 hr at 37° C. The results were subject to analysis of variance isolating sums of squares due to differences between treatments, hours, ejaculates and their interactions. The second order interaction has been used to calculate the standard error, used in the *t*-test.

High dilution and severe washing have similar effects on the cation concentration of spermatozoa. Both procedures caused a considerable increase in sodium and a marked depletion of potassium, which was often accentuated by incubating the spermatozoa for 3 hr. There was also a decrease in the calcium and magnesium concentration of bull spermatozoa as previously observed at lower dilutions.

DISCUSSION

These studies confirm the finding of Dott & White (1964) that a 1:2 dilution of ram semen in sodium phosphate buffer causes a marked fall in the potassium concentration within the spermatozoa. A similar effect is seen with bull spermatozoa and with both species there is an efflux of calcium and magnesium as

well as potassium. From the results of Quinn *et al.* (1965) on the distribution of potassium, calcium and magnesium between spermatozoa and seminal plasma, it is clear that the concentration of all three cations will be considerably less in the surrounding medium than in the spermatozoa after a 1:2 dilution. The efflux of these ions is, therefore, in the direction of the lessening concentration gradient.

The compensatory movement of sodium into the spermatozoa is also along a lessening concentration gradient, since the sodium concentration of the diluents greatly exceeds that of the undiluted ram and bull spermatozoa (Quinn *et al.*, 1965).

TABLE 3

CONCENTRATION (mg/100 g) OF CATIONS IN SPERMATOZOA AFTER WASHING FOUR TIMES AND 1:100 DILUTION. SODIUM PHOSPHATE DILUENT OF pH = 7.0 WAS USED AND ANALYSES ARE MADE IMMEDIATELY AND AFTER 3 hr

Species	Time	Treatment	Sodium	Potassium	Calcium	Magnesium
Ram (four replicates)	0 hr	Undiluted	103	87	6.0	7.7
		Washed	361**	22**	4.2	7.5
		Diluted	308**	32**	6.1	11.0
	3 hr	Undiluted	117	91	5.7	8.0
		Washed	380**	5**	3.7	4.1
		Diluted	479**	4**	3.5	3.6
			S.E. = 33.4	S.E. = 6.3	S.E. = 1.78	S.E. = 1.93
	0 hr	Undiluted	132	230	39.3	18.1
		Washed	187	40**	6.0**	9.2**
		Diluted	258	13**	2.8**	4.7**
Bull (five replicates)	3 hr	Undiluted	131	195	39.0	28.6
		Washed	224	33**	3.3**	6.3**
		Diluted	318	46**	8.3**	15.0*
			S.E. = 89.2	S.E. = 13.4	S.E. = 5.90	S.E. = 4.94

* $P < 0.05$; ** $P < 0.01$ (significantly different from undiluted).

The marked depletion of potassium in spermatozoa on diluting 1:100 or exhaustive washing is consistent with reports that such treatment has a detrimental effect on the motility of ram and bull spermatozoa. This effect can be reduced by including potassium in the sodium phosphate diluent (White, 1953b; Dott & White, 1964). Potassium must, however, fall to very low levels (see Table 3) before motility is impaired. Milder treatments, such as a 1:2 dilution of ram semen in sodium phosphate, reduce the potassium concentration of the spermatozoa from 141 to 118 mg/100 g (Table 1), and yet motility and impedance frequency change are not greatly affected by this treatment (Dott & White, 1964).

Although phosphate is not specific in its effect on the spermatozoa, the cation concentration of the diluted cells was to some extent dependent on the diluent used. Sodium influx into ram spermatozoa was greater in phosphate than in tris buffer, due probably to a lower sodium concentration in the latter diluent (386 mg/100 g compared with 240 mg/100 g). A more puzzling observation was the increase in the calcium content of ram spermatozoa produced by tris at pH 7.0 but not at 8.5.

The injurious effect of high concentrations of phosphate on the oxidative metabolism of bull spermatozoa has been previously reported (Bishop & Salisbury, 1955; Salisbury & Nakabayashi, 1957; White, 1957; Lodge, Salisbury, Schmidt & Graves, 1963), but the adverse effect on motility has apparently escaped notice. A 1:2 dilution of ram and bull semen in the veronal buffer immediately halved the motility of the spermatozoa. Barbiturates have been shown to uncouple oxidative phosphorylation in liver mitochondria (Aldridge & Parker, 1960), but the adverse effect on the motility of spermatozoa in this instance is probably more direct. At lower concentrations veronal has been used as a diluent for spermatozoa (Blackshaw, 1953a; Wales & White, 1958c).

The observation that acid conditions decrease the loss of potassium from spermatozoa may be of some physiological significance in maintaining intracellular potassium levels and may, in part, explain why there is little loss of potassium when undiluted semen samples are incubated for several hours.

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