THE EFFECT OF ALKALI METAL, MAGNESIUM, AND CALCIUM IONS ON THE MOTILITY OF FOWL SPERMATOZOA

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Summary

Potassium (5–45 mM), magnesium (1·5–13·5 mM), and calcium (0·3–2·7mM) chlorides each increase the viability of fowl spermatozoa *in vitro* when added to a diluent composed of 0·02M sodium phosphate buffer, 0·5 per cent. sodium chloride, and 1·5 per cent. glucose.

Additive effects were given by potassium in combination with magnesium or calcium but motility in the diluent containing magnesium and calcium was less than would be expected from the sum of their separate effects.

Calcium ions in concentrations greater than 0·3 mM caused agglutination of the spermatozoa.

Washing twice or more was harmful to fowl spermatozoa and the effect of potassium was relatively greater on the washed than the unwashed cells.

Rubidium ions were as effective as potassium ions in stimulating motility, caesium ions were ineffective, and lithium and ammonium ions were toxic.

Potassium levels in excess of 180 mM depressed the motility of fowl spermatozoa and diluents composed entirely of potassium salts are therefore undesirable.

I. Introduction

Potassium, magnesium, and calcium occur in appreciable quantities in semen (see Mann 1954) and attention has recently been drawn to the effect of these ions on spermatozoa (White 1956).

The importance of potassium for the normal functioning of ram and bull spermatozoa can be readily demonstrated by comparing their viability after repeated washing in potassium-containing and potassium-free media (Lardy and Phillips 1943; White 1953*a*, 1953*b*; Blackshaw 1953*a*, 1953*b*). Viability remains high in the solution containing potassium but the spermatozoa become immotile after 3 hr at 37°C in its absence. Studies of the capacity of the rarer alkali metals to replace potassium in washed ram and bull spermatozoa show an interesting gradation in biological properties which can be readily correlated with their sequence in the periodic tables (White 1953*c*).

According to Lardy and Phillips (1943) magnesium improves the motility and glycolysis of washed bull spermatozoa; calcium on the other hand has been found to decrease the viability of ram and bull spermatozoa (Lardy and Phillips 1943; Blackshaw 1953*a*).

There appears to be little or no information on the ionic requirements of fowl spermatozoa and it is the purpose of this paper to report studies on the effect of the alkali metals, magnesium, and calcium ions on the spermatozoa of this species.

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II. Materials and Method

Fowl semen was obtained by abdominal massage (Burrows and Quinn 1939). Only apparently normal specimens of good initial motility were employed.

Unwashed spermatozoal suspensions were prepared by diluting 0·5 ml of semen with 4·5 ml of diluent in a graduated centrifuge tube, and washed spermatozoa were prepared by centrifuging this suspension at 1500 r.p.m. (about 300 *g*) for 10 min, the supernatant being drawn off and replaced by diluent after each centrifuging and the spermatozoa re-dispersed by sucking up and down in a wide-bore pasteur pipette fitted with a rubber teat. Centrifuged but unwashed spermatozoal suspensions were prepared by centrifuging the diluted (1 in 10) semen and re-dispersing the cells after each centrifuging without removing the supernatant.

Table 1

CONCENTRATIONS OF POTASSIUM, MAGNESIUM, AND CALCIUM IN DILUENTS

Metal	Level 1 (mM)	Level 2 (mM)	Level 3 (mM)
Potassium	5	15	45
Magnesium	1·5	4·5	13·5
Calcium	0·3	0·9	2·7

The diluted semen was pipetted into small tubes and kept at room temperature. For the determination of motility, a drop of spermatozoal suspension was placed on a glass slide and examined under the microscope. Motility was scored by the system of Emmens (1947). Full motility was rated as four and complete immotility as zero, but in presenting the results the actual scores have been multiplied by 4, since quarter-grades were frequently used. The sum of the motility scores multiplied by 4 for each ejaculate over the experimental period has been used as unit observation (see Emmens 1948) in the analyses of variance which are presented in summary form.

All diluents were isotonic and of pH 7·0. They were prepared from A.R. chemicals and stored in a deep-freeze cabinet at −80°C between experiments. The control diluent had the following composition: 0·02M sodium phosphate buffer, 0·5 per cent. sodium chloride, 1·5 per cent. glucose. Potassium, magnesium, and calcium were added as the chloride salts at the concentrations shown in Table 1 and the sodium chloride content adjusted to keep the diluent isotonic.

III. Results

(*a*) *Preliminary Experiments*

The technique of washing mammalian spermatozoa has been widely used in studying their metabolism and ionic requirements. The damage caused to ram and bull spermatozoa by the process has been investigated by White (1953*d*).

As no studies of the effect of washing on avian spermatozoa have been reported, the effect of washing and of sedimentation by centrifuging on fowl spermatozoa was studied by comparing the motility of the unwashed cells with that of washed suspensions and with spermatozoa centrifuged without washing. The washing and centrifuging procedures were repeated in each case twice and four times. Two diluents used were (i) the control and (ii) the control plus level 1 (see Table 1)

TABLE 2

EFFECT OF WASHING AND CENTRIFUGING ON THE MOTILITY INDICES OF FOUR FOWL EJACULATES

Treatment	Diluent A (control)					Diluent B (control + level 1 of potassium and magnesium)					Totals
	1	2	3	4	Total	1	2	3	4	Total	
Diluted	45	58	62	55	220	58	61	63	63	245	465
Washed twice	25	40	25	10	100	45	60	53	29	187	287
Centrifuged twice	39	55	62	52	208	52	58	60	56	226	434
Washed four times	22	35	17	6	80	43	59	53	23	178	258
Centrifuged four times	32	48	61	45	186	45	60	62	54	221	407
Totals	163	236	227	168	794	243	298	291	225	1057	1851

Analysis of Variance

Source of Variation	Degrees of Freedom		Variance Ratio	
Between diluents	1		86·5**	
Between ejaculates	3		27·1**	
Between treatments	4		53·0**	
Washing *v.* centrifuging		1		136·8**
Washing twice *v.* washing four times		1		2·7
Centrifuging twice *v.* centrifuging four times		1		2·3
Treatment *v.* controls		1		70·2**
Interactions				
Diluent × treatment	4		8·6**	
Diluent × ejaculate	3		0·5	
Treatment × ejaculate	12		5·7**	
Residual	12		20	

** $P < 0{\cdot}01$.

of potassium and magnesium since both ions had proved beneficial to mammalian spermatozoa, particularly after washing. Motility was scored at 2, 4, 8, and 16 hr from the start of each test.

The results for four ejaculates and the analysis of variance are given in Table 2. Motility in the diluent containing potassium and magnesium was significantly better,

especially after washing. Partitioning of the treatment sum of squares showed that washing was more harmful than merely centrifuging. Clearly the damage done

TABLE 3

EFFECT OF POTASSIUM, MAGNESIUM, AND CALCIUM ON THE MOTILITY OF UNWASHED AND TWICE-WASHED FOWL SPERMATOZOA

Each value represents the mean motility index for six ejaculates

Ions Added	Unwashed				Washed				Grand Mean
	Level 1	Level 2	Level 3	Mean	Level 1	Level 2	Level 3	Mean	
Nil	42·0	44·3	44·2	43·5	23·5	24·2	21·8	23·2	33·4
Potassium	46·2	48·7	41·7	45·6	29·7	27·8	22·7	26·7	36·1
Magnesium	50·7	48·0	50·0	49·6	29·5	29·8	32·3	30·6	40·1
Calcium	47·5	45·5	49·0	47·3	25·5	27·0	27·0	26·5	36·9
Potassium, magnesium	50·8	51·3	47·8	50·0	32·9	37·7	34·0	34·8	42·4
Potassium, calcium	50·0	50·0	53·0	51·0	30·8	33·8	31·3	32·0	41·5
Magnesium, calcium	49·0	51·0	51·7	50·6	28·2	30·2	28·5	28·9	39·8
Potassium, magnesium, calcium	52·3	52·0	51·2	51·8	35·2	33·0	38·3	35·5	43·7

Analysis of Variance

Source of Variation	D.F.	Variance Ratio	Source of Variation	D.F.	Variance Ratio
Effect of potassium	1	31·0**	Interactions (*Continued*)		
Effect of magnesium	1	45·4**	Potassium × levels	2	1·0
Effect of calcium	1	14·4**	Magnesium × levels	2	0·1
Effect of washing	1	866·4**	Calcium × levels	2	2·0
Between levels	2	0·2	Potassium × ejaculate	5	3·9**
Between ejaculates	5	109·8**	Magnesium × ejaculate	5	2·7*
Interactions			Calcium × ejaculate	5	5·3**
Potassium × magnesium	1	0·2	Washing × ejaculate	5	24·3**
Potassium × calcium	1	1·2	Levels × ejaculate	10	0·8
Magnesium × calcium	1	8·0**	Levels × washing	2	0·2
Potassium × washing	1	7·1**	Second-order interactions	86	29·5†
Magnesium × washing	1	1·1	Higher-order interactions	146	22·6
Calcium × washing	1	0·9			

* $P < 0·05$. ** $P < 0·01$.

† The second-order interaction mean square is significantly larger than the higher-order interaction mean square ($F = 1·3$, $P = 0·05$) and has been used as error term.

to fowl spermatozoa by repeated washing is not due to the mechanical effect of centrifuging alone and is partly offset by the addition of potassium and magnesium.

(*b*) *Systematic Studies of Potassium, Magnesium, and Calcium*

In view of the beneficial action of the diluent containing potassium and magnesium, especially after washing, more detailed factorial studies were made of these two ions and of calcium.

Potassium, magnesium, and calcium ions were added to the control diluent at levels 1, 2, and 3 (Table 1) so that within each level all possible combinations of the three ions were tried but no between-level combinations were made. In all diluents containing the highest concentration of calcium a slight opalescence was noted due to the precipitation of calcium phosphate.

Half of each pooled ejaculate was partitioned between the diluents to give a 1 in 10 dilution of semen. The other half was diluted 1 in 10 with control diluent and washed twice before partitioning. Motility was scored at 4, 8, 16, and 24 hr from the start of each test. The results for six replicates and the analysis of variance are presented in Table 3. The SILLIAC electronic computer was used for this analysis. As Bartlett's test showed that the variances of the second-order interactions were homogeneous ($\chi^2 = 20 \cdot 5$, 19 degrees of freedom, $P = 0 \cdot 3$), the pooled variance was used as error term since it was significantly greater than the higher-order interaction mean square.

Potassium, magnesium, and calcium all cause a highly significant increase in motility but the effects of magnesium are greater than those of potassium or calcium. Additive effects were given by potassium in combination with magnesium, calcium, or magnesium plus calcium but the motility in the diluent containing magnesium and calcium was significantly less than would be expected from the sum of their separate effects and was almost equal to the motility in magnesium alone. No significant variation in motility was seen between the three metal levels. As in the previous experiment, washing had a detrimental effect on motility and the beneficial action of potassium was greater on washed than on unwashed cells.

In the course of this experiment, marked agglutination of the spermatozoa was noted in tubes containing either level 2 or 3 of calcium. Thus in four ejaculates agglutination was found in eight of the 24 tubes containing calcium singly or in combination at level 2, and in 19 of the 24 tubes at level 3. Agglutination occurred as frequently in the unwashed as in the washed samples and neither potassium nor magnesium influenced the effect.

(*c*) *Potassium Toxicity*

Further studies were undertaken to determine the toxic level of potassium for fowl spermatozoa. It was impossible to obtain toxic levels of magnesium and calcium since the phosphate buffer diluent was saturated with these ions at the highest level previously tried.

Completely replacing the sodium chloride in the control diluent with potassium chloride had no detrimental effect on motility. Toxic levels of potassium were, however, obtained by using potassium phosphate buffer in the diluent and then replacing the sodium chloride by increasing amounts of potassium chloride. The final concentrations of potassium in the three diluents prepared in this way were

150, 180, 210 mM. Tests with five ejaculates (Table 4) showed a linear fall in motility with increasing potassium concentrations.

(*d*) *Comparison of Alkali Metals*

Since low concentrations of potassium increased the motility of fowl spermatozoa it was of interest to compare the effectiveness of the other alkali metals. The ammonium ion was also included in these tests as it substitutes for potassium in some biological systems (Boyer, Lardy, and Phillips 1942, 1943; Muntz 1947).

TABLE 4

EFFECT OF HIGH POTASSIUM CONCENTRATIONS ON THE MOTILITY OF FOWL SPERMATOZOA

Potassium Concentration (mM)	Ejaculate					Totals
	1	2	3	4	5	
0	62	60	63	62	64	311
150	58	59	62	61	62	302
180	56	56	49	54	57	272
210	49	45	43	50	41	228
Totals	225	220	217	227	224	1113

Potassium, lithium, rubidium, calcium, and ammonium chlorides were added to the control diluent to give a final concentration of 5 mM. Fowl spermatozoa were washed twice in the control diluent. The final volume of the suspension was adjusted to that of the original semen and aliquots were mixed with nine parts of the diluents containing the alkali metals. In order to produce a greater and more rapid effect, the diluted sperm suspensions were kept at 37°C for the first 2 hr before being scored at room temperature.

Motility was observed at 2, 4, 6, 8 hr from the start of each test and Table 5 shows the result for six replications. Potassium and rubidium significantly increased motility but the ammonium ion depressed it; lithium had no effect under these conditions (Table 5). If, however, the sperm suspensions were kept at room temperature throughout the test, motility in the control diluent was maintained better and it was possible to demonstrate a toxic effect of lithium.

IV. DISCUSSION

Washing harms fowl spermatozoa to about the same extent as it does ram and bull spermatozoa (White 1953*d*), but they pack down into a particularly solid mass on centrifuging and are much more difficult to resuspend. This may account for the harmful effect of centrifuging fowl spermatozoa four times. Centrifuging cannot be the most important factor in washing, however, since the motility of centrifuged spermatozoa was much better than that of the washed cells, particularly at the start of experiments. As the beneficial effect of potassium was relatively greater

after washing, loss of this ion from the cell may account for some of the damage. The motility of spermatozoa washed in the potassium-containing diluents was, however, still below the centrifuged control and other substances such as cytochrome *c* and glyceraldehyde 3-phosphate dehydrogenase are probably also leached from the spermatozoa (Mann 1951; Smith, Mayer, and Merilan 1957).

TABLE 5

EFFECT OF ALKALI METAL IONS AND AMMONIUM IONS ON THE MOTILITY OF TWICE-WASHED FOWL SPERMATOZOA

Ions Added	Ejaculate						Totals
	1	2	3	4	5	6	
Nil	26	19	10	17	54	20	146
Potassium	50	51	32	35	59	49	276
Lithium	14	16	11	18	44	15	118
Rubidium	37	39	30	40	56	49	251
Caesium	31	25	14	37	42	29	178
Ammonium	10	11	6	24	30	17	98

Analysis of Variance

Source of Variation	Degrees of Freedom		Variance Ratio	
Between ejaculates	5		16·7**	
Between diluents	5		24·7**	
Sodium *v.* potassium		1		40·2**
Sodium *v.* lithium		1		1·9
Sodium *v.* rubidium		1		26·3**
Sodium *v.* caesuim		1		2·4
Sodium *v.* ammonium		1		5·6*
Residual	25		35	

* $P < 0·05$. ** $P < 0·01$.

These experiments clearly establish the importance of potassium and magnesium for the maintenance of full viability of fowl spermatozoa and in contrast to its action on ram and bull spermatozoa (Lardy and Phillips 1943, Blackshaw 1953*b*) calcium also proved beneficial. Diluents containing the lowest level of potassium and magnesium should prove quite satisfactory for the spermatozoa of this species. It is inadvisable to include calcium in such diluents since it causes agglutination in concentrations above 0·3 mM. Fowl spermatozoa, like those of the ram and bull (White 1953*c*) are very tolerant to high potassium levels but diluents composed entirely of potassium salts are undesirable.

Since the beneficial action of potassium was not influenced by magnesium or calcium it seems likely that the divalent ions act at a different site from potassium

in the spermatozoa. Magnesium and calcium, on the other hand, may have a similar role since the results suggest that they are interchangeable in their effect on motility. Similar results have been obtained by Wales and White (1958) using dog spermatozoa. Potassium and magnesium are important for several different reactions in the glycolytic cycle and may exert their stimulating action on fowl spermatozoa in this way.

The reaction of fowl spermatozoa to the rarer alkali metals is similar to that of the ram, bull, and dog (White 1953*c*; Wales and White 1958) and the ability of rubidium to replace potassium in spermatozoa would seem to be a widespread phenomenon. Lithium on the other hand appears to be generally spermicidal; it depresses the motility of human as well as ram, bull, and dog spermatozoa (MacLeod, Swan, and Aitken 1949; White 1953*c*; Wales and White 1958). Fowl spermatozoa are unique in that their motility is depressed by concentrations of ammonium ions innocuous to other species (White 1953*c*).

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