EFFECTS OF SOME BUFFERS AND INORGANIC AND ORGANIC SODIUM SALTS IN SYNTHETIC DILUENTS FOR THE STORAGE OF RAM SPERMATOZOA AT 37 OR 5°C

By K. R. Lapwood*† and I. C. A. Martin*

[Manuscript received 28 September 1971]

Abstract

Buffers, inorganic and organic salts, salts of volatile fatty acids, and lyophilized non-dialysable skim milk solids were tested in saline–sugar diluents used for storage of ram spermatozoa at 37 and 5°C.

When tested at 5°C, phosphate, veronal–HCl, and Tris–HCl buffers improved the value of diluents which did not contain milk solids. At 37°C, and at 5°C in diluents containing milk solids, these three buffers depressed the activity of spermatozoa, while a fourth, Tris–maleate, was toxic in all experiments.

20 mM sodium sulphate, in diluents without milk solids, and 20 mM sodium tetraborate were valuable additions to diluents used at 37 and 5°C, respectively, but scores of activity of spermatozoa were reduced to zero when the level of the latter salt was increased to 40 mM. Diluents containing 20 or 40 mM lactate, pyruvate, succinate, or citrate generally depressed the activity of spermatozoa stored at either temperature, succinate and citrate being particularly deleterious at 5°C.

When sodium salts of volatile fatty acids (C_1–C_6) were used in diluents at concentration between 1 and 20 mM, inclusions of up to 5 mM were beneficial at 5°C. At 37°C, none of these salts improved the value of the diluents, and sodium acetate was the only one which did not depress activity when tested at a level of 20 mM.

The survival of spermatozoa was improved by the addition of 0·5% lyophilized non-dialysable skim milk solids to the diluent.

I. Introduction

Diluents for the storage of semen should be buffered against changes in pH (Melrose 1962; Mann 1964). Phosphate buffers commonly were employed by early workers (Anderson 1945) but were replaced by mixtures of citrate and egg yolk as diluents for bull semen because an optically clearer medium resulted (Salisbury, Fuller, and Willett 1941). However, fertility results indicated that phosphate diluents were superior to those containing citrate (Campbell and Edwards 1955). Other buffer systems which have proved satisfactory in diluents for bull semen are: bicarbonate (Kampschmidt *et al.* 1951; Kampschmidt, Mayer, and Herman 1953; VanDemark and Sharma 1957); and Tris (Bomstein and Steberl 1959). Veronal–HCl buffers were equally as suitable as phosphate for inclusion in diluents for semen of ram, rabbit, and bull (Blackshaw 1953) and dog (Wales and White 1958).

* Department of Veterinary Physiology, University of Sydney, Sydney, N.S.W. 2006.

† Present address: Department of Physiology and Anatomy, Massey University, Palmerston North, New Zealand.

Although the inclusion of citrate in semen diluents has been studied widely (Emmens and Robinson 1962; Melrose 1962; Mann 1964), there have been few reports on the inclusion of other products of metabolism. The unsatisfactory results obtained from citrate diluents used for storage of ram spermatozoa at 5°C (Martin, unpublished data), and at −79°C (Jones and Martin 1965), as well as for storage of dog spermatozoa at −79°C (Martin 1963), indicates the need for further study of the functions of citrate in diluents.

Sodium sulphate was often used in earlier studies with bull, stallion, boar, and rabbit semen (Anderson 1945), and ram semen (Panyseva 1940), while Yoshiola, Inudo, and Torizuka (1951) found that the longevity and fertility of ram and goat semen was improved by use of a boric acid–sodium bicarbonate diluent.

Shannon (1962) reported that addition of acetic, propionic, butyric, caproic, and octanoic acids to diluents for bull semen improved viability during storage at 37 and 5°C; best results were obtained with 0·025% (v/v) caproic acid. There was a significant improvement in the fertility of semen stored in this diluent for 24–36 hr before insemination.

The inclusion of lyophilized non-dialysable skim milk solids in semen diluents has been shown to improve spermatozoal viability (Choong and Wales 1963; Jones 1965; Jones and Martin 1965).

In this paper the experiments were designed to study the storage of ram spermatozoa at 37 and 5°C in synthetic diluents containing varying levels of the following groups of compounds: buffers, inorganic and organic sodium salts, and sodium salts of volatile fatty acids. In addition, experiments 1 and 2 tested the inclusion of 0·5% (w/v) lyophilized non-dialysable skim milk solids in the diluents.

II. Materials and Methods

Semen was collected from Merino rams by electrical stimulation (Blackshaw 1954), and only samples with good motility (3·5 or 4·0 on the scale of Emmens 1947), and with a high percentage of motile spermatozoa, were used.

The methods of dilution, incubation at 37°C, chilling to and storage at 5°C, and of scoring spermatozoal survival, have been described previously (Lapwood and Martin 1966). The dilution rates were 70-fold in experiments 1 and 2, and 40-fold in experiments 3 and 4. The periods of incubation/storage for each experiment are shown in the tables of results.

The diluents used in experiment 1 contained 185 mM fructose and up to 62 mM sodium chloride. The buffers (pH 7·0): Na_2HPO_4–NaH_2PO_4, Tris–HCl, Tris–maleate; and veronal–HCl were included in some diluents, reciprocally replacing isosmotic amounts of sodium chloride. In experiment 2 the diluents contained: 74 mM fructose, 20 mM Na_2HPO_4–NaH_2PO_4 buffer, and up to 85 mM NaCl. The following sodium salts were included in some diluents, again reciprocally replacing sodium chloride: sulphate, tetraborate, pyruvate, lactate, succinate, and citrate. Where indicated in the tables of results, 0·5% (w/v) lyophilized non-dialysable skim cow milk was included in diluents tested in these two experiments.

For experiments 3 and 4 diluents contained 31 mM NaCl, 20 mM Na_2HPO_4–NaH_2PO_4 buffer, up to 185 mM sugar, 0·8% (w/v) milk solids, and 17 mM fructose. The sugars used were glucose for 37°C studies, and arabinose for 5°C studies (following Lapwood and Martin 1966), and were reciprocally replaced by isosmotic amounts of sodium chloride, or the sodium salts of the volatile fatty acids: formic, acetic, propionic, butyric, valeric, and caproic. Because most of the volatile fatty acid salts were quite hygroscopic, estimations of the sodium content of standard solutions were made on an atomic absorption spectrophotometer, and where necessary solution strengths were adjusted to give the required salt concentrations.

Five hundred i.u./ml each of sodium penicillin-G and dihydrostreptomycin sulphate were added to all diluents.

Four replicates, each using ejaculates from different rams, were completed in each experiment.

Experimental Design and Analysis

Separate analyses of variance were computed for motility scores and for the angular transformations of the percentage of motile spermatozoa recorded for each temperature of storage in the experiment. In experiment 3, where the test samples were examined after 8 and 24 hr of incubation at 37°C, the analysis was treated as in a split-plot experiment where the effect of time was considered within-samples.

In both experiments 1 and 2, the buffer systems or anions and the levels of these compounds used were incorporated into single factors having respectively 20 and 13 levels. The forms of contrasts used for the first, or diluent, factor in the analyses of variance, are shown in Tables 1 and 2. Table 1 also gives the set of contrasts used in both experiments 3 and 4.

TABLE 1

SETS OF ORTHOGONAL COEFFICIENTS USED FOR THE BETWEEN-DILUENT CONTRASTS PARTITIONED IN THE ANALYSES OF VARIANCE OF THE DATA OF EXPERIMENTS 2, 3, AND 4

Expt. No.	Contrast	Diluent code*						
		1	2	3	4	5	6	7
2	*A.* Control diluent contrasted with all other preparations							
	B. Organic *v.* inorganic anions		2	2	−1	−1	−1	−1
	C. Pyruvate and lactate *v.* succinate and citrate		0	0	−1	−1	1	1
	D. Sulphate *v.* borate		−1	1	0	0	0	0
	E. Pyruvate *v.* lactate		0	0	−1	1	0	0
	F. Succinate *v.* citrate		0	0	0	0	−1	1
	The remaining 6 degrees of freedom were used in individual tests of the effects of change in concentration of each anion							
3 and 4	*A.* Chloride *v.* volatile fatty acids (VFA)	6	−1	−1	−1	−1	−1	−1
	B. VFA C_2 *v.* C_1	0	1	−1	0	0	0	0
	C. VFA C_3 *v.* lower	0	1	1	−2	0	0	0
	D. VFA C_4 *v.* lower	0	1	1	1	−3	0	0
	E. VFA C_5 *v.* lower	0	1	1	1	1	−4	0
	F. VFA C_6 *v.* lower	0	1	1	1	1	1	−5

* Refer to Tables 3, 4, and 5 for key to diluent composition for each experiment.

Both the chemistry of the compounds and the plan to test simultaneously a number of unrelated substances which might have been similar in their effects on spermatozoa, lead to the construction of the matrices of orthogonal contrasts. The particular grouping of contrasts between buffers arose as a sequel to earlier experiments in which phosphate had been compared with veronal and, separately, with Tris–HCl. The sequence of contrasts used in experiment 2 followed the division of the anions into a broad grouping of organic and inorganic compounds. Then, within the organic group, substances arising in the glycolytic or citric acid cycle metabolic pathways were paired. An overall factor to describe level of compound has little meaning within such experimental plans, so that the effects of concentration were only studied within each compound.

The analyses of variance of experiments 3 and 4 were similar to those summarized for experiment 1 as all first-order interactions were isolated and the variance of second-order, or higher

TABLE 2

EXPERIMENT 1: MEAN MOTILITY SCORES AND MEAN PERCENTAGES OF MOTILE SPERMATOZOA AFTER STORAGE AT 37 AND 5°C

Results for 37°C are means of the total hourly scores from 1–6 hr, and those for 5°C are means of the scores after 1 and 3 days

	Buffer concn. (mM)	37°C		5°C	
		Motility	% Motile	Motility	% Motile
I. Buffer and level					
No buffer	—	2·97	55·4	0·92	22·2
Phosphate	7	3·06	57·1	1·12	24·6
	14	2·96	55·2	0·96	25·3
	21	2·89	56·1	1·12	28·1
	28	2·63	52·2	1·12	27·8
Tris–HCl	7	2·79	53·3	0·90	18·1
	14	3·11	55·8	1·15	25·9
	21	2·71	51·0	0·90	18·4
	28	2·49	45·2	1·03	19·6
Tris–maleate	7	2·15	45·7	0·31	3·7
	14	1·95	45·5	0·40	4·6
	21	1·60	36·1	0·47	6·5
	28	1·67	38·3	0·37	4·0
Veronal–HCl	7	2·74	51·9	0·90	22·1
	14	2·63	49·9	0·97	21·8
	21	2·36	50·5	1·22	30·3
	28	2·00	45·0	1·18	30·6
II. Lyophilized milk preparation	0	2·38	46·2	0·52	8·4
	0·5% (w/v)	2·79	54·9	1·26	31·6

Summary of Analyses of Variance

Source of variation	D.F.	Variance ratios for 37°C		Variance ratios for 5°C	
		Motility	% Motile	Motility	% Motile
I. Buffers:					
Buffer type	4				
No buffer *v.* buffer	1	46·6***	19·89***	0·38	3·80
Phosphate *v.* veronal	1	22·56***	14·21***	0·40	0·02
Tris–HCl *v.* Tris–maleate	1	120·39***	35·37***	71·47***	82·59***
Phosphate and veronal *v.* Tris–HCl and Tris–maleate	1	33·68***	25·80***	56·41***	124·69***
Effects of concn. of individual buffers	15				
Phosphate, linear	1	6·01*	0·49	0·12	1·23
Tris–HCl, linear	1	8·80**	8·14**	0·30	0·07
Tris–HCl, cubic	1	2·20	0·30	3·17	4·80*
Tris–maleate, linear	1	10·32**	8·76**	0·30	0·07
Veronal, linear	1	18·96***	3·52	5·76*	9·44**
Remainder	10	0·91	0·89	0·70	0·71
II. Lyophilized milk preparation	1	13·14*	12·54*	30·33*	56·22**
III. Ejaculates	3	8·69***	7·53***	5·51**	6·12**
Interactions					
I×II	19	0·25	0·33	0·33	0·84
I×III	57	1·02	1·10	0·91	1·16
II×III	3	4·90**	5·51**	8·77***	7·86***
Residual	57	*17·29*†	*1637*†	*1·33*†	*192·98*†

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$. † Residual variance.

interactions was used as the error term. In the simpler design for the studies of spermatozoa stored at 5°C in experiment 2, the diluent × replicate interaction was used as the estimate of error variance.

In both experiments 3 and 4 the series of salts of the volatile fatty acids from C_1 to C_6 was studied and here a series of contrasts were used to test the effects of one of the series against all members with a lower molecular weight. As these compounds are closely related, the effects of level of substances under test was examined using a separate factor "level". In addition all single degree of freedom contrasts within the interaction of compounds × level were generated by the direct product of the compound and level matrices of contrasts.

The significance of contrasts made within factor I of each of experiments 2, 3, and 4 is shown at the foot of each of the Tables 3, 4, and 5.

Error (residual) variance is shown in italic script and the degrees of freedom are given in parentheses.

III. Results

Experiment 1 was a $(5\times4)\times2\times4$ design in which the factors were: (1*a*) no buffer or phosphate, Tris–HCl, Tris–maleate, or veronal–HCl buffer, (1*b*) 7, 14, 21, or 28 mM buffer and, to preserve the symmetry of the experimental design, four samples of diluent without buffer were prepared for each ejaculate; (2) presence or absence of 0·5% (w/v) lyophilized, non-dialysable milk solids; and (3) four ejaculates (replicates). Results, and the summary of the analyses of variance, are shown in Table 2.

Significant results for observations made after incubation of samples at 37°C were:

(1) The mean scores of activity were reduced as a result of part replacement of sodium chloride by buffer.

(2) Diluents containing phosphate buffer were better than those containing veronal.

(3) Activity of spermatozoa in diluents containing the Tris–maleate buffer was poorer than in those containing Tris–HCl.

(4) The low scores from the Tris–maleate diluents were the main reasons for the significantly poorer mean scores from the two Tris buffers, than from diluents with phosphate and veronal buffers.

(5) Increasing the levels of buffers caused a decline in viability of spermatozoa. In diluents containing the Tris buffers scores of both motility and percentage motile showed this effect, but when phosphate and veronal buffers were used the decline in viability was significant only for motility scores.

(6) Inclusion of 0·5% (w/v) milk solids in the diluents resulted in a significant improvement in viability.

Significant results at 5°C were:

(1) Mean results from diluents containing the Tris buffers were again lower than from diluents which contained phosphate or veronal buffers. This effect was very pronounced when Tris–maleate was used.

(2) The scores of percentage motile observed in diluents containing Tris–HCl just fitted a cubic response curve ($P < 0{\cdot}05$).

(3) In contrast to the results obtained at 37°C, the viability of spermatozoa was improved when the level of veronal buffer was increased.

(4) Inclusion of 0·5% (w/v) milk solids was again beneficial.

Experiment 2, a $(2\times6+1)\times4$ factorial (Table 3) tested diluents in which sodium chloride was replaced by 20 or 40 mM of the following sodium salts: sulphate, tetraborate, pyruvate, lactate, succinate, or citrate. The compounds were assumed to be completely ionized, and as the number of ions varied it was necessary to replace

TABLE 3

EXPERIMENT 2: MEAN MOTILITY SCORES AND MEAN PERCENTAGE OF MOTILE SPERMATOZOA AFTER STORAGE FOR 6 HR AT 37°C OR 6 DAYS AT 5°C

	Level of anion (mM)	Level of NaCl (mM)	37°C		5°C	
			Motility	% Motile	Motility	% Motile
I. Anion tested:						
1. Control (chloride)	—	85	2·43	46·2	1·25	22·5
2. Sulphate	20	55	2·37	42·5	0·62	10·0
	40	25	2·62	48·7	0·25	3·7
3. Borate	20	55	2·43	41·2	1·37	36·2
	40	25	1·25	20·0	0·00	0·0
4. Pyruvate	20	65	2·31	38·7	0·87	15·0
	40	45	2·00	36·8	1·12	12·5
5. Lactate	20	65	2·37	44·3	0·87	12·5
	40	45	2·06	36·8	0·75	10·0
6. Succinate	20	55	2·12	32·5	0·00	0·0
	40	25	1·18	21·2	0·00	0·0
7. Citrate	20	45	2·12	36·8	0·37	3·7
	40	5	1·56	26·2	0·25	2·5
II. Lyophilized milk preparation	0·0% (w/v)		1·44	24·2	Milk solids present in all diluents	
	0·5% (w/v)		2·66	48·4		
	Contrast (Table 1)					
	A		n.s.	n.s.	**	**
	B		n.s.	n.s.	n.s.	n.s.
	C		n.s.	**	***	***
	D		***	**	n.s.	n.s.
	E		n.s.	n.s.	n.s.	n.s.
	F		n.s.	n.s.	n.s.	n.s.
	Error variance (36)		*12·77*	*465·3*	*2·55*	*196·3*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

varying levels of sodium chloride to maintain isosmotic conditions. The effect of including 0·5% (w/v) lyophilized non-dialysable skim milk solids was again tested, at 37°C, but milk solids were present in all samples stored at 5°C.

Significant results at 37°C were:

(1) The mean percentage of motile spermatozoa observed in diluents containing pyruvate and lactate was higher than that in diluents containing succinate and citrate (contrast *C*, Tables 1 and 3).

(2) An increase in the level of sodium borate from 20 to 40 mM caused a significant decline in activity, which also meant that the mean response to borate was significantly lower than that for sulphate (contrast *D* and levels within borate).

(3) Addition of 0·5% (w/v) milk solids was beneficial.

Significant results at 5°C were:

(1) The mean scores calculated from diluents in which sodium chloride was partially replaced with other salts were poorer than from the control diluent containing 85 mM sodium chloride (contrast *A*).

(2) Of the organic salts tested, pyruvate and lactate were not as detrimental as were succinate and citrate (contrast *C*).

(3) Viability was again lower in diluents containing 40 mM rather than 20 mM borate.

TABLE 4

EXPERIMENT 3: VIABILITY OF RAM SPERMATOZOA AFTER INCUBATION FOR 8 AND 24 HR AT 37°C, OR FOR 6 DAYS AT 5°C

Results are the means of response over all three levels of each salt in the diluent

	8 hr (37°C)		24 hr (37°C)		6 days (5°C)	
	Motility	% Motile	Motility	% Motile	Motility	% Motile
I. Salt						
1. Sodium chloride	3·04	59·2	2·00	43·3	1·71	40·0
2. Sodium formate	2·25	51·7	1·29	39·2	1·58	49·2
3. Sodium acetate	2·87	53·2	1·83	41·2	1·83	40·8
4. Sodium propionate	2·79	53·3	1·37	35·8	1·50	32·9
5. Sodium butyrate	2·46	43·3	0·91	23·3	1·83	40·8
6. Sodium valerate	2·54	51·7	1·33	34·2	1·92	40·0
7. Sodium caproate	2·17	42·5	0·95	25·0	1·92	46·7

Contrast	Significance of effects at 37°C		Significance of effects at 5°C	
	Motility	% Motile	Motility	% Motile
I. Salts				
A	***	***	n.s.	n.s.
B	***	n.s.	n.s.	*
C	n.s.	n.s.	n.s.	**
D	***	***	n.s.	n.s.
E	n.s.	n.s.	n.s.	n.s.
F	***	***	n.s.	n.s.
II. Levels of salts				
5 mM *v.* 20 mM	***	n.s.	***	***
Error variance (36)	*0·70*	*44·00*	*0·38*	*14·40*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Experiment 3 was a $7 \times 3 \times 4$ design in which the factors were: (i) partial replacement of the glucose or arabinose of the diluent by sodium chloride or the sodium salts of the volatile fatty acids: formic, acetic, propionic, butyric, valeric,

caproic; (ii) levels of these salts of 5, 10, or 20 mM; and (iii) four ejaculates. In the experiment at 37°C the effect of incubation for 8 and 24 hr was also tested. Results are shown in Table 4, and significant effects at 37°C were:

(1) Diluents containing sodium chloride were better on average, than those containing salts of fatty acids (contrast *A*).

(2) Higher motility scores were seen in diluents containing sodium acetate, than in those containing sodium formate (contrast *B*).

(3) Mean scores of activity where formate, acetate or propionate was present were greater than when the diluent contained butyrate (contrast *D*).

(4) Poorer viability scores were observed from diluents with caproate than the mean scores from diluents containing the sodium salts of the shorter chain volatile fatty acids (contrast *F*).

(5) Over all diluents, better motility scores were obtained when glucose was replaced by only 5 mM, rather than 20 mM, of these salts.

(6) Both scores of motility and of percentage of motile spermatozoa fell significantly in the interval from 8 to 24 hr incubation and the rate of decline in activity of spermatozoa was not significantly affected by the presence of any individual volatile fatty acid.

Significant results at 5°C were:

(1) More spermatozoa were motile in the diluents containing formate than in those containing acetate (contrast *B*). However, the mean percentage of motile spermatozoa in diluents containing these two salts was greater than from those containing sodium propionate (contrast *C*).

(2) Again, diluents containing 5 mM of these salts were superior to those with 20 mM.

The same design was used in experiment 4, summarized in Table 5, in which sodium salts of volatile fatty acids, and sodium chloride again replaced glucose or arabinose, in studies at 37°C and 5°C, respectively. In this experiment they were used at 1, 2, and 4 mM, but the main effect of level of volatile fatty acid salt was non-significant.

Significant results at 37°C were:

(1) The motility score for spermatozoa in diluents containing sodium valerate was higher than the mean score, calculated from the observation on diluents containing formate, acetate, propionate, and butyrate (contrast *E*).

Significant results at 5°C were only detected in the analysis of the data for percentage motile and these were:

(1) Samples in which arabinose was replaced by sodium chloride contained fewer motile spermatozoa than those in which the sodium salts of fatty acids replaced arabinose (contrast *A*).

(2) Diluents containing formate were better than those containing acetate; these together were in turn superior to diluents containing propionate (contrasts *B* and *C*).

Considering only diluents containing sodium caproate, the best percentage of motile spermatozoa was seen when the level of this salt was 2 mM ($P < 0{\cdot}05$, using a separate comparison within the data on the effects of the inclusion of caproate).

TABLE 5

EXPERIMENT 4: MEAN MOTILITY SCORES AND MEAN PERCENTAGES OF MOTILE SPERMATOZOA AFTER 8 HR AT 37°C, AND 6 DAYS AT 5°C

Results are means of response to three levels (1, 2, and 4 mM) of each volatile fatty acid used

	37°C		5°C	
	Motility	% Motile	Motility	% Motile
I. Salt				
1. Sodium chloride	2·50	56·7	2·04	38·3
2. Sodium formate	2·33	58·3	2·12	51·7
3. Sodium acetate	2·50	60·8	2·12	45·8
4. Sodium propionate	2·50	57·5	2·04	44·2
5. Sodium butyrate	2·50	56·7	2·00	45·8
6. Sodium valerate	2·66	60·0	2·21	45·0
7. Sodium caproate	2·37	55·0	2·12	46·7

Contrast	Significance of effects at 37°C		Significance of effects at 5°C	
	Motility	% Motile	Motility	% Motile
A	n.s.	n.s.	n.s.	***
B	n.s.	n.s.	n.s.	*
C	n.s.	n.s.	n.s.	*
D	n.s.	n.s.	n.s.	n.s.
E	*	n.s.	n.s.	n.s.
F	n.s.	n.s.	n.s.	n.s.
Error variance (36)	*0·37*	*14·79*	*0·22*	*17·07*

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

IV. DISCUSSION

In the absence of a theory which would help to predict the effects of various anions, both organic and inorganic, in diluents for the preservation of living cells, it is necessary to undertake experiments to screen the response of spermatozoa to different levels of these compounds at several temperatures. Thus we have continued with the type of experimental design used previously (Lapwood and Martin 1966) investigating on this occasion a variety of substances which, for convenience, have been grouped according to chemistry or function. All of the substances tested were introduced into the diluent by reciprocal replacement of the sodium chloride or sugar element of the

solutions. This technique minimizes the risk of confounding the effects of an increase in osmolarity of the diluent with those of the substances under test but the response to the substance is confused with the simultaneous reduction in the other compound. Whereas the systematic appraisal of sugars in diluents (Lapwood and Martin 1966) led to the identification of the value of glucose and arabinose as major components of diluents for ram spermatozoa, the results of the experiments reported here have not suggested any diluent structure better than 185 mM sugar, 31 mM sodium chloride, and 20 mM phosphate buffer. Indeed, taking the alternative view possible where reciprocal replacement has been used, reduction in the level of chloride in the diluent mixtures of experiment 2 and of the content of sugar in the diluents used in experiments 3 and 4 could be a component of all the diluent effects observed.

Of the buffers tested at 37°C in experiment 1, phosphate was the most satisfactory, but caused a decline in motility at higher levels. Similar depression of motility, and also of metabolic activity, due to the inclusion of phosphate in semen diluents has been observed by several authors (Bishop 1954; Dauzier, Thibault, and Wintenberger 1954; Mann and White 1957; Salisbury and Nakabayashi 1957) while O'Shea and Wales (1964) showed that phosphate increased the deleterious effects of high levels of potassium (64 mM). However, other authors have obtained satisfactory motility and fertility results from the use of semen of various species diluted in solutions containing phosphate (Emmens 1947; Campbell and Edwards 1955; Wales and White 1963; Dott 1964; Dott and White 1964).

Generally the results of studies at 5°C in experiment 1 were similar to those obtained at 37°C; however, the buffers did not have an overall depressing effect on viability, and phosphate and veronal buffers were of equal value. Although a decline in survival of spermatozoa occurred in diluents containing milk solids and 28 mM phosphate, Tris–HCl, or veronal–HCl buffers, the use of high levels of these buffers improved survival in diluents without milk solids. It is apparent that under these conditions of storage, the optimal buffer level was influenced by the inclusion of milk solids.

The motility and endogenous respiration of bull spermatozoa were inhibited by 10 mM maleate in studies by Lardy and Phillips (1943*a*, 1943*b*), and it is considered that this compound was the main cause of the poor results observed from diluents containing Tris-maleate buffer.

Schindler and Amir (1961) found that a diluent containing 0·1% lactic acid maintained the motility of ram spermatozoa at satisfactory levels, while several authors have reported that citrate-containing diluents are also satisfactory for storage of ram spermatozoa (Koger 1951; Salamon and Robinson 1962; Feredean and Brăgăru 1963). However, other reports indicate that citrate diluents are suboptimal for spermatozoa of this species (Schindler and Amir 1961; Dzajant 1965; Jones and Martin 1965). In more recent studies (Martin, unpublished data) it was found that citrate, at levels of 10 and 20 mM, reduced the viability of spermatozoa stored in a fructose synthetic diluent at 5°C; this effect was particularly marked in diluents containing the higher citrate level together with 0·8% (w/v) milk solids. Dauzier, Thibault, and Wintenberger (1954) reported that although optimal motility was obtained in a yolk–citrate diluent, the fertility of semen stored in this diluent was

only satisfactory if used within 2 hr of collection, and even then was lower than that obtained from semen stored in a saline diluent.

Scott, White, and Annison (1962) reported an increase in oxygen uptake by ram spermatozoa resulting from the use of diluents containing 18 mM of salts of volatile fatty acids, and Shannon (1962) observed that inclusion of low levels of volatile fatty acids improved the viability of diluted bull semen. In experiments 3 and 4 in this paper there was no improvement in the viability of ram spermatozoa incubated at 37°C in diluents containing salts of the C_1–C_6 acids. In the last experiment the addition of levels of between 1 and 4 mM of volatile fatty acids to the diluent was beneficial to spermatozoa stored at 5°C. However, unlike bull spermatozoa kept in similar conditions (Shannon 1962), ram spermatozoa did not show greatest response, within this series of compounds tested, to the presence of caproate. Thus at neither temperature was any relationship detected between the value of these substances in diluents for storage of ram semen, and their value for increasing oxygen uptake (Scott, White, and Annison 1962), or their molecular weight.

V. Acknowledgments

We are grateful to Professor C. W. Emmens for his advice on and criticism of this paper. This research was performed with the aid of grants from the Australian Wool Board.

VI. References

Anderson, J. (1945).—"The Semen of Animals and its Use for Artificial Insemination." (Imp. Bur. Anim. Breed. Genetics: London.)

Bishop, M. W. H. (1954).—Some aspects of the dilution effect in bovine spermatozoa. *Stud. Fert.* **6**, 81.

Blackshaw, A. W. (1953).—The effects of potassium and calcium salts on the motility of ram, rabbit and bull spermatozoa. *J. Physiol.* **120**, 645.

Blackshaw, A. W. (1954).—A bipolar rectal electrode for the electrical production of ejaculation in sheep. *Aust. Vet. J.* **30**, 249.

Bomstein, R. A., and Steberl, E. A. (1959).—Preservation of washed bovine spermatozoa in a synthetic medium at room temperature. *Expl. Cell Res.* **18**, 217.

Campbell, R. C., and Edwards, J. (1955).—The effects on conception rates of semen diluents containing citrate or phosphate buffer with all combinations of sulphanilamide, streptomycin and penicillin. *J. agric. Sci.* **46**, 44.

Choong, C. H., and Wales, R. G. (1963).—The use of various diluents for deep-freezing bull spermatozoa. *Aust. J. biol. Sci.* **16**, 896.

Dauzier, L., Thibault, C., and Wintenberger, S. (1954).—Conservation du sperme de belier apres dilution et maintein de son pouvoir fecondant. *Annls. Endocr.* **15**, 341.

Dott, H. M. (1964).—Results of inseminations with ram spermatozoa in different media. *J. Reprod. Fertil.* **8**, 157.

Dott, H. M., and White, I. G. (1964).—Effect of potassium on ram spermatozoa studied by a flow dialysis technique. *J. Reprod. Fertil.* **7**, 127.

Dzajant, H. (1965).—More progeny from valuable rams. *Ovcevodstvo* **11** (1), 21. (*Anim. Breed. Abstr.* **33**, No. 1376.)

Emmens, C. W. (1947).—The motility and viability of rabbit spermatozoa at different hydrogen-ion concentrations. *J. Physiol.* **106**, 471.

Emmens, C. W., and Robinson, T. J. (1962).—Artificial insemination in the sheep. In "The Semen of Animals and Artificial Insemination". (Ed. J. P. Moule.) (Commonw. Agric. Bur.: Farnham Royal.)

FEREDEAN, T., and BRĂGĂRU, F. (1963).—The preservation of ram semen by freezing to −79°C. *Lucr. stiint. Inst. Cerc. zooteh.* **21**, 357. [*Anim. Breed. Abstr.* **33**, No. 1377.]

JONES, R. C. (1965).—Studies of the handling and insemination of ram semen. Ph.D. Thesis, University of Sydney.

JONES, R. C., and MARTIN, I. C. A. (1965).—Deep-freezing ram spermatozoa: The effects of milk, yolk–citrate, and synthetic diluents containing sugar. *J. Reprod. Fertil.* **10**, 413.

KAMPSCHMIDT, R. F., MAYER, D. T., and HERMAN, H. A. (1953).—Viability of bull spermatozoa as influenced by various sugars and electrolytes in the storage medium. Res. Bull. Univ. Mo. Agric. Exp. Sta. No. 519.

KAMPSCHMIDT, R. F., MAYER, D. T., HERMAN, H. A., and DICKERSON, G. E. (1951).—Viability of bull spermatozoa as influenced by electrolyte media. *J. Dairy Sci.* **34**, 45.

KOGER, M. (1951).—Storage, dilution and use of ram semen in artifical breeding of sheep. Bull. New. Mex. agric. Exp. Stn No. 366.

LAPWOOD, K. R., and MARTIN, I. C. A. (1966).—The use of monosaccharides, disaccharides, and trisaccharides in synthetic diluents for storage of ram spermatozoa at 37 and 5°C. *Aust. J. biol. Sci.* **19**, 655.

LARDY, H. A., and PHILLIPS, P. H. (1943*a*).—Inhibition of sperm respiration and reversibility of effects of metabolic inhibitors. *J. biol. Chem.* **148**, 333.

LARDY, H. A., and PHILLIPS, P. H. (1943*b*).—Inhibition of sperm glycolysis and reversibility of the effects of metabolic inhibitors. *J. biol. Chem.* **148**, 343.

MANN, T. (1964).—"The Biochemistry of Semen and of the Male Reproductive Tract." (Methuen and Co., Ltd.: London.)

MANN, T., and WHITE, I. G. (1957).—Glycerol metabolism of spermatozoa. *Biochem. J.* **65**, 634.

MARTIN, I. C. A. (1963).—The deep-freezing of dog spermatozoa in diluents containing skim milk. *Res. vet. Sci.* **4**, 315.

MELROSE, D. R. (1962).—Artificial insemination. In "The Semen of Animals and Artificial Insemination". (Ed. J. P. Moule.) (Commonw. Agric. Bur.: Farnham Royal.)

O'SHEA, T., and WALES, R. G. (1964).—Effects of potassium on ram spermatozoa during chilling to and storage at 5°C. *J. Reprod. Fertil.* **8**, 121.

PANYSEVA, L. V. (1940).—Iskusstvennoe osemenenie ovec malymi dozami razbavlennoi spermy. *Trudy Lab. iskusst. osemen. Zivotn.* (*Mosk.*) **1**, 296. (*Anim. Breed. Abstr.* **13**, No. 38.)

SALAMON, S., and ROBINSON, T. J. (1962).—Studies on the artificial insemination of Merino sheep. II. The effects of semen diluents and storage on lambing performance. *Aust. J. agric. Res.* **13**, 271.

SALISBURY, G. W., FULLER, H. K., and WILLETT, E. L. (1941).—Preservation of bovine spermatozoa in yolk–citrate diluents and field trials from its use. *J. Dairy Sci.* **24**, 905.

SALISBURY, G. W., and NAKABAYASHI, N. T. (1957).—Effect of phosphate and chloride ions on aerobic metabolism of bovine spermatozoa. *J. exp. Biol.* **34**, 52.

SCHINDLER, H., and AMIR, D. (1961).—Longevity of ram sperm in various diluents and at different dilution rates. *J. agric. Sci.* **56**, 183.

SCOTT, T. W., WHITE, I. G., and ANNISON, E. F. (1962).—Oxidation of short chain fatty acids (C_1–C_8) by ram, bull, dog and fowl spermatozoa. *Biochem. J.* **83**, 392.

SHANNON, P. (1962).—Effect of additions of volatile fatty acids on the viability and fertility of diluted bovine semen. *Nature, Lond.* **196**, 1225.

VANDEMARK, N. L., and SHARMA, U. D. (1957).—Preliminary fertility results from the preservation of bovine semen at room temperatures. *J. Dairy Sci.* **40**, 438.

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.

WALES, R. G., and WHITE, I. G. (1963).—Viability of diluted dog spermatozoa *in vitro*. *J. Reprod. Fertil.* **5**, 67.

YOSHIOKA, Z., INUDO, Y., and TORIZUKA, T. (1951).—Experiments on the storage of semen and the insemination of stored semen in sheep and goats. Bull. natn. Inst. agric. Sci., Tokyo. Ser. G. No. 1. p. 53.