THE USE OF DIMETHYL SULPHOXIDE, GLYCEROL, AND RECONSTITUTED SKIM MILK FOR THE PRESERVATION OF RAM SPERMATOZOA

II. THE INFLUENCE OF DILUENT COMPOSITION AND PROCESSING TIME DURING FREEZING TO —79°C WITH DIMETHYL SULPHOXIDE OR GLYCEROL OR BOTH COMPOUNDS

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Summary

Reconstituted skim milk diluents containing dimethyl sulphoxide or glycerol or both substances were used to deep-freeze ram spermatozoa to —79°C. Very poor revival (less than 10%) followed freezing in dimethyl sulphoxide. Small improvements in revival were obtained by adding 2% (w/v) fructose to the diluent and shortening the equilibration time to 10 min before freezing. The inclusion of 1·5% (v/v) dimethyl sulphoxide in a diluent containing 7% (v/v) glycerol improved revival.

Hypertonic diluents were better than isotonic diluents for freezing ram spermatozoa. Replacement of the milk solids by fructose, while the tonicity of the freezing diluent remained constant, did not significantly affect revival. Spermatozoa revived equally well after storage for 5 hr at 5°C before freezing either in the presence of glycerol (equilibrated spermatozoa) or its absence (aged spermatozoa).

I. Introduction

Although bull semen in an egg yolk–citrate diluent containing 7·5% (v/v) glycerol and 1·25% (w/v) fructose has satisfactory fertility (Emmens and Martin 1957; Martin and Emmens 1958), a lambing rate of only 5–10% followed insemination of ewes with ram semen similarly processed by Emmens and Blackshaw (1955). *In vitro* studies (Blackshaw 1960) indicated that milk may replace egg yolk–citrate as a diluent for freezing, but this substitution does not improve fertility (First, Sevinge, and Henneman 1961).

A review by Emmens and Robinson (1962) describes diluents used for the storage of ram spermatozoa at 30, 5, and —79°C. Emmens and Blackshaw (1956) and Polge (1957) reviewed reports of deep-freezing of mammalian spermatozoa using glycerol, and refer to earlier reviews by Parkes (1945) and Polge and Parkes (1952). Smith and Polge (1950*b*) examined a number of polyhydric alcohols and their derivatives, sugars, colloids, and other agents. Only ethylene and propylene glycol gave revival of fowl spermatozoa approaching that obtained with glycerol, which they used successfully to freeze bull and goat semen (Smith and Polge 1950*a*). Using human, ram, bull, and rabbit semen, Emmens and Blackshaw (1950) tested a number of sugars (pentoses, hexoses, and di- and trisaccharides) and a variety of mono- or polyhydric alcohols alone and as combinations, as well as inositol and

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urea, and found that a mixture of an alcohol and a sugar gave superior results. The best combinations were glycerol (7·5–10% v/v) and arabinose (1·25% w/v) for the ram and bull and similar quantities of ethylene glycol and a sugar for rabbit semen.

Lovelock (1954) showed that glycerol was not alone in its protective action during the freezing of erythrocytes. A number of neutral solutes of low molecular weight, including methanol, acetamide, formamide, and glyceryl monoacetate also protected erythrocytes during freezing and thawing. Later, Lovelock and Bishop (1959) reported that dimethyl sulphoxide was superior to glycerol in protecting erythrocytes, but inferior when used for freezing bull spermatozoa. Ashwood-Smith (1961*a*, 1961*b*) used dimethyl sulphoxide to freeze mouse bone marrow cells successfully.

Jones (1965) showed that dimethyl sulphoxide did not contribute to the tonicity of a diluent for ram spermatozoa and, although it appeared less toxic to spermatozoa than glycerol during incubation at 30°C, it was more toxic than glycerol at 5°C. Reconstituted skim milk powder (9% w/v) was best for storage of spermatozoa at either 5 or 30°C, so in the following studies it is considered to be an isotonic solution for spermatozoa. The following experiments examine the composition of a reconstituted skim milk diluent and the value of dimethyl sulphoxide as a protective agent during the freezing of ram spermatozoa.

II. Materials and Methods

(*a*) *Diluents and Processing of Spermatozoa*

Ram semen was collected by electro-ejaculation using the multipolar electrode described by Blackshaw (1954). Samples showing good activity, i.e. strong wave motion [a score of 3·5–4·0 (Emmens 1947)] were initially diluted for use in these experiments within 40 min of collection. In all experiments, semen was diluted 20-fold at 30°C in the initial diluent containing 9% (w/v) reconstituted skim milk powder and 0·3% (w/v) fructose. After chilling to 5°C over 2 hr in 1-ml volumes this was further diluted 1 : 1 in a single addition (except in experiment 1) with the diluent containing the protective agent. This diluent at 5°C had the same concentration of milk solids as the original diluent at 30°C except in experiment 6 where levels of milk solids were varied. It had twice the final concentration of protective agent and fructose so that mixing with equal volumes of diluted semen gave the desired concentration of protective agent and fructose. Final fructose levels varied in experiments 2, 3, and 6, but were 1·25% (w/v) in all other experiments. Spermatozoa were stored at 5°C (aged spermatozoa) for 1·5 hr and equilibrated for 15 min in experiment 5, but were equilibrated for 0·5 hr in presence of glycerol without aging in the other experiments unless otherwise determined by experimental design. The diluted semen was frozen in 1-ml volumes in all experiments. Freezing rate was varied in experiments 3 and 4 but otherwise semen was frozen in a device patterned on that described by Polge and Lovelock (1952) which gave a freezing rate of 0·5–1 degC/min to −10 to −15°C after which the rate increased to about 3 degC/min.

All frozen semen was stored for more than 24 hr at −79°C and thawed in a water-bath at 37°C. The thawed spermatozoa in experiments 1, 5, and 6 were concentrated after scoring by centrifuging at 700 *g* for 10 min. The supernatant was removed and the spermatozoa resuspended and incubated for 1 hr in the resuspending media described below.

Incubation diluent.—This was made up of a mixture of 4 parts by volume of Krebs–Henseleit–Ringer (Mann 1954) to 1 part of 0·1M sodium phosphate buffer, with 0·3% (w/v) added fructose and 0·5% (w/v) of non-dialysable solids from skim (cow) milk.

TABLE 1

EFFECT OF LEVEL OF DIMETHYL SULPHOXIDE AND GLYCEROL ON THE STAINING REACTION ON THAWING OF SPERMATOZOA DEEP-FROZEN TO −79°C

Results are means from four ejaculates

Glycerol (% v/v)	No. of Spermatozoa Unstained (%)	Dimethyl Sulphoxide (% v/v)	No. of Spermatozoa Unstained (%)
3·0	36·3	4·0	22·9
5·0	31·6	6·0	21·8
7·0	24·8	8·0	25·4
9·0	24·6	10·0	10·6

(*b*) *Scoring and Analyses of Results*

The design, analyses, scoring, and coding techniques were similar to those used by Jones (1965). In addition to scores of motility and percentage of motile spermatozoa at thawing, congo red–nigrosin stains were made (Blackshaw 1955) in experiments 1, 4, 5, and 6. 100 spermatozoa were observed per smear and classified as stained or unstained. Counts of percentage unstained spermatozoa and scores of percentage motile spermatozoa were transformed to angles for the analyses of variance. The method of describing experimental contrasts as "linear" (*L*) and "quadratic" (*Q*) and the corresponding partition of interaction variance using orthogonal polynomials followed that of Cochran and Cox (1957).

III. RESULTS

Since only a small proportion of spermatozoa survived deep-freezing in dimethyl sulphoxide, the results of experiments 1, 2, and 3 are presented in a summarized form (Tables 1, 2, and 3). Analyses of variance of the data in these experiments gave similar error mean squares as in the other experiments which are reported more fully in this paper. The significance of mean effects is presented in Tables 2 and 3, whilst the significant interactions are described below.

Experiment 1 was a $2^2 \times 4^2$ factorial design in which the protective action during freezing of dimethyl sulphoxide (4, 6, 8, and 10% v/v) and glycerol (3, 5, 7, and 9% v/v) were compared, the total volume of the solution containing the protective agent was added as one single 1 : 1 dilution at 5°C or was added in four successive dilutions (i.e. 0·1, 0·2, 0·2, and 0·5 of the volume of first-stage diluent) over 0·5 hr. Revival

TABLE 2

EXPERIMENT 2: MEAN EFFECTS OF LEVELS OF DIMETHYL SULPHOXIDE AND FRUCTOSE IN DILUENTS HYPER- AND ISOTONIC RELATIVE TO 9% (w/v) RECONSTITUTED POWDERED MILK ON THE REVIVAL OF RAM SPERMATOZOA AFTER DEEP-FREEZING TO −79°C

Treatment	Mean Motility Score	Mean Score of Spermatozoa Motile (%)
Control [glycerol, 7·5% (v/v); fructose, 1·25% (w/v); reconstituted powdered milk, 9% (w/v)]	2·92	27·5
Fructose (% w/v): 0·5	0·85	3·1
1·25	0·92	3·8
2·0	1·33	6·5
P (linear):	<0·01	<0·01
Dimethyl sulphoxide (% v/v): 3·0	0·67	2·1
6·0	1·48	7·4
9·0	0·96	3·8
P (quadratic):	<0·001	<0·001
Diluent isotonic	0·83	3·2
Diluent hypertonic (fructose added)	1·24	5·7
P:	<0·01	<0·01
Ejaculates: 1	0·94	3·6
2	0·92	2·3
3	1·25	7·4
4	1·03	4·4
P:	n.s.	<0·05

after freezing in glycerol was poor (10–40% motile). However, even when spermatozoa frozen in dimethyl sulphoxide were resuspended in a diluent free of this compound after thawing, only 1–5% were motile. An analysis of variance revealed no significant difference in the responses obtained after the two methods of adding the protective agent, but in the data for percentage of unstained spermatozoa there was a significant interaction ($P<0·01$) of compound and dose level (Table 1). This meant that an equal proportion of spermatozoa frozen in either 7% glycerol or 8% dimethyl sulphoxide were penetrated by congo red stain after thawing, whilst at all other levels of protective agent, glycerol was superior to dimethyl sulphoxide.

The factors studied in experiment 2, a $2\times3^2\times4$ factorial design, are shown in Table 2. No treatment combination produced a revival rate as good as that obtained with the control diluent which contained glycerol ($P<0\cdot01$). Overall, 2·0% fructose and 6·0% dimethyl sulphoxide were the best levels of each factor tested. These diluents were hypertonic. Three interactions are significant: the quadratic × quadratic component of the interaction of fructose and dimethyl sulphoxide was significant for measures of percentage number of spermatozoa motile.

TABLE 3

EXPERIMENT 3: MEAN EFFECTS OF FREEZING RATE TO −40°C, LEVEL OF DIMETHYL SULPHOXIDE, AND EQUILIBRATION TIME ON THE REVIVAL OF RAM SPERMATOZOA DEEP-FROZEN TO −79°C

Treatment	Mean Motility Score	Mean Score of Spermatozoa Motile (%)
Rate of freezing to −40°C: 1 degC/min	1·56	13·3
2 degC/min	1·42	13·9
4 degC/min	1·61	17·2
P (linear):	n.s.	<0·05
Protective agent:		
(1) Glycerol (7·5% v/v)+fructose (1·25% w/v)	2·46	37·3
(2) Dimethyl sulphoxide (3·0% v/v)+fructose (2·0% w/v)	1·39	7·6
(3) Dimethyl sulphoxide (6·0% v/v)+fructose (2·0% w/v)	1·19	6·1
(4) Dimethyl sulphoxide (9·0% v/v)+fructose (2·0% w/v)	1·13	8·3
P for (1) *v.* mean of (2), (3), and (4):	<0·001	<0·001
P for (2) *v.* (4):	<0·05	n.s.
Equilibration time: 0·5 hr	1·53	13·3
5·0 hr	1·53	16·4
Ejaculates: 1	1·48	11·5
2	1·58	18·0
3	1·35	9·6
4	1·71	19·8
P:	<0·05	<0·001

With 1·25% fructose present, the mean scores for samples frozen in 6·0% dimethyl sulphoxide were much the same as for samples frozen in 3·0 and 9·0% (i.e. mean of 4·3 *v.* 3·5% motile), but at the other fructose levels of 0·5 and 2·0%, 6% dimethyl sulphoxide gave better results than the average of the other two levels (i.e. mean of 10·9 *v.* 2·9%). Both measures of response showed a significant quadratic × linear interaction of fructose and tonicity. Thus, for example, in the isotonic diluent the mean percentage of spermatozoa motile after freezing in 1·25% fructose is 1·4 and the corresponding value for spermatozoa frozen in 0·5 and 2·0% fructose is larger (i.e. 5·2); however, in the hypertonic diluent these values were very much the same (i.e. 6·1 and 5·9). The third significant interaction is the linear × linear component

of the interaction of dimethyl sulphoxide and tonicity for scores of percentage motile spermatozoa. In this case, scores were similar for isotonic and hypertonic diluents containing 3·0% dimethyl sulphoxide (means of 1·9 and 2·3% motile) but spermatozoa survived freezing better in a hypertonic than in an isotonic diluent containing 9·0% dimethyl sulphoxide (mean score of 1·9 *v.* 6·2).

Table 3 shows the factors and mean effects of a $2\times3\times4^2$ factorial experiment (experiment 3). None of the semen samples frozen in dimethyl sulphoxide revived as well as samples frozen in glycerol. Motility scores on thawing were better when semen was frozen in 3%, than in 6 or 9% dimethyl sulphoxide and the greatest

TABLE 4

EXPERIMENT 4: EFFECTS OF EQUILIBRATION, FREEZING RATE, AND LEVELS OF GLYCEROL AND DIMETHYL SULPHOXIDE ON THE REVIVAL OF SPERMATOZOA DEEP-FROZEN TO −79°C

Treatment	Mean Motility Score	Mean Score of Spermatozoa Motile (%)	Mean No. of Spermatozoa Unstained (%)
Protective agent			
6·0% (v/v) glycerol	2·98	33·5	50·3
9·0% (v/v) glycerol	2·79	27·9	35·5
6·0% (v/v) dimethyl sulphoxide	1·17	4·3	32·8
9·0% (v/v) dimethyl sulphoxide	1·13	3·8	24·2
Freezing rate			
1 degC/min	2·02	18·3	36·3
4 degC/min	2·01	16·4	35·0
Equilibration time			
10 min	2·06	18·8	38·1
40 min	2·11	18·2	34·3
70 min	1·84	15·2	34·5
Ejaculates			
1	2·13	20·4	26·5
2	1·44	9·0	13·3
3	2·27	39·9	52·6
4	2·23	13·5	50·4

proportion of motile spermatozoa was observed on thawing when semen was frozen at the rate of 4 degC/min. The significant interaction of protective agent and equilibration for both measures of response shows that a period of equilibration decreased the survival of spermatozoa frozen in glycerol (e.g. mean percentage motile score of 43·8 *v.* 30·8) but had no effect on spermatozoa frozen in dimethyl sulphoxide (mean percentage motile score of 7·4 *v.* 7·3%). However, this equilibration effect with glycerol was only observed in two of the four ejaculates used in the experiment.

The effects of shorter equilibration times in dimethyl sulphoxide or glycerol were studied in experiment 4—a $2^3\times3\times4$ factorial (Tables 4 and 5). Again poor revival followed freezing in dimethyl sulphoxide. With more than 10 min equilibration in either protective agent, the proportion of thawed spermatozoa stained

by congo red significantly increased. This effect was not apparent for other measures of response, but there was a significant linear×linear interaction of freezing rate and equilibration time in the scores of percentage motile spermatozoa (Fig. 1). After 10 min equilibration, a freezing rate of 4 degC/min was better than 1 degC/min. The slower rate was best when a 70-min equilibration period was used. All treatment responses showed a significant ejaculate interaction for at least one measure of response. This meant that the rank order of each ejaculate's mean response varied

TABLE 5

SUMMARY OF ANALYSES OF VARIANCE OF THE DATA IN EXPERIMENT 4

Source of Variation	Degrees of Freedom	Variance Ratios		
		Motility Score	Percentage Motile	Percentage Unstained
Protective agents (*A*)	3			
(i) Dimethyl sulphoxide *v.* glycerol	1	412·94***	447·34***	100·87***
(ii) Common linear regression	1	1·79	4·74*	63·65***
(iii) Departure from parallelism	1	0·72	2·05	3·49
Freezing rate (*B*)	1	0·01	0·85	2·57
Equilibration (*C*)	2			
10 min *v.* 70 min (*L*)	1	3·21	2·22	6·13*
40 min *v.* mean of 10 and 70 min (*Q*)	1	2·40	0·60	3·14
Ejaculates (*D*)	3	20·83***	36·68***	190·32***
Interactions				
A×*B*	3	0·25	2·50	2·13
A×*C*	6	1·26	0·73	1·28
B×*C*	2			
L×*L*	1	0·36	14·03***	0
L×*Q*	1	0·74	0·48	0·02
A×*D*	9	2·78*	8·80***	1·36
B×*D*	3	0·53	2·29	3·30*
C×*D*	6	1·78	2·43*	0·93
Residual mean square	56†	0·70	27·11	21·57

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

† The degrees of freedom have been reduced by one because a missing value was inserted in the data according to the method described by Cochran and Cox (1957).

with treatment. Thus, for example, the ejaculate which survived best after freezing in dimethyl sulphoxide (for motility and percentage motile scores) ranked only third in survival when frozen in glycerol.

A mean of 2·7% of spermatozoa frozen in dimethyl sulphoxide were motile after resuspension and incubation for 1 hr at 37°C, so that only the data relating to spermatozoa frozen in glycerol and incubated after thawing were analysed. Since only ejaculate differences were significant, the analyses are not presented in Table 5.

In experiment 5 (a 3×4^2 factorial), spermatozoa were frozen in diluents containing glycerol alone or in combination with dimethyl sulphoxide. Scores were made on thawing and after the spermatozoa were incubated for 1 hr at 37°C (Table 6). In the analyses of the latter data, the total variance was less than was obtained with scores immediately on thawing, but a similar partition of this variance was made for the two sets of data (Table 7). On thawing, scores of motility and percentage of motile spermatozoa were largest when spermatozoa were frozen in either 5 or 7% (v/v) glycerol, the difference in response obtained for these two levels being small. However, a smaller proportion of spermatozoa frozen in either 3 or 5% (v/v) glycerol was stained by congo red–nigrosin than those frozen in 7% (v/v) glycerol. Generally, increasing concentrations of dimethyl sulphoxide in the diluent decreased revival. The linear×linear and linear×cubic partition of the interaction between levels of dimethyl sulphoxide and glycerol were just significant ($P<0\cdot05$). Thus, best revival was observed when spermatozoa were frozen in a mixture of 7% (v/v) glycerol and 1·5% (v/v) dimethyl sulphoxide. This advantage persisted after incubation for 1 hr at 37°C, but was not significant.

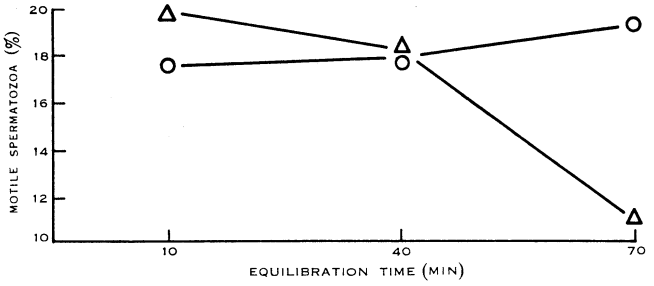

Fig. 1.—Interaction of freezing rate and equilibration time on the percentage of motile ram spermatozoa after deep-freezing. ○ 1 degC/min. △ 4 degC/min.

Tables 8 and 9 describe the results and analyses of a $2^3\times3\times4$ factorial experiment in which the factors were: (*a*) tonicity of the freezing diluent relative to 0·154M NaCl (= 1·0) : 1·0, 1·2, and 1·4; (*b*) factor adding to the tonicity of the final diluent: thus, fructose was added to an 8% (w/v) milk diluent or milk was added to a diluent containing 1·25% (w/v) fructose to the required tonicity; (*c*) glycerol levels of 4 and 8% (v/v); (*d*) the glycerol-containing diluent was either added immediately the semen had cooled to 5°C (equilibrated) or it was added 4·5 hr later (aged), the semen being frozen after storage for 5 hr at 5°C; (*e*) replications using ejaculates from four rams. A hypertonic freezing diluent was much better than an isotonic diluent for all measures of response and the cause of the increased tonicity was not important. No advantage was gained by increasing the diluent tonicity from 1·2 to 1·4. Scores of motility and percentage motile were highest when spermatozoa were frozen in 8 rather than 4% (v/v) glycerol. Although this difference was highly significant in the analyses of scores made immediately on thawing, it was not significant for scores made after incubation for 1 hr at 37°C. Samples frozen in 4% (v/v) glycerol showed more unstained spermatozoa at thawing

than did those frozen in 8% (v/v) glycerol. Spermatozoa survived better, as indicated by all scores except motility scores at thawing, when aged than when equilibrated, but the difference was not statistically significant.

IV. Discussion

The poor revival obtained for ram spermatozoa frozen in dimethyl sulphoxide constrasts with its value as a protective agent during the freezing of other biological tissues (Ashwood-Smith 1961*a*, 1961*b*), but confirms Lovelock and Bishop's (1959) report on its use for freezing bull spermatozoa. Most workers who have described

Table 6

Experiment 5: effect of combinations of dimethyl sulphoxide and glycerol on the revival of deep-frozen ram spermatozoa, scored after thawing and after incubation for 1 hr at 37°C

Glycerol Concn. (% v/v)	Dimethyl Sulphoxide Concn. (% v/v)	Mean Motility Score		Mean Score of Spermatozoa Motile (%)		Mean No. of Thawed Spermatozoa Unstained (%)
		Thawed	Incubated	Thawed	Incubated	
3·0	0	2·38	2·00	13·8	11·3	45·0
3·0	1·5	2·38	1·75	13·8	7·5	51·5
3·0	3·0	2·50	1·88	16·3	10·0	48·3
3·0	4·5	2·25	1·63	15·0	7·5	42·3
5·0	0	2·88	2·13	37·5	12·5	49·0
5·0	1·5	2·88	2·13	30·0	11·3	48·3
5·0	3·0	2·75	2·50	31·3	17·5	52·0
5·0	4·5	2·25	1·75	16·3	8·8	38·8
7·0	0	2·63	1·88	31·3	13·8	40·0
7·0	1·5	3·00	2·25	45·0	21·3	38·8
7·0	3·0	2·38	2·00	10·0	12·5	35·5
7·0	4·5	2·25	1·50	11·3	11·3	28·8
Ejaculates: 1		2·75	2·21	24·2	15·0	46·0
2		2·54	1·92	25·4	13·8	51·1
3		2·58	2·00	26·3	10·8	35·5
4		2·29	1·67	21·7	8·8	40·8

the protective action of dimethyl sulphoxide during freezing have used reticulo-endothelial cells as the test material—either the undifferentiated heterogeneous cells in the bone marrow (Ashwood-Smith 1961*a*, 1961*b*; Persidsky and Richards 1963) or mature myeloid cells, i.e. erythrocytes (Lovelock and Bishop 1959), which are not nucleated. In the latter case, the degree of haemolysis on thawing was the measure of response rather than transfusion studies of viability. Thus it is possible that dimethyl sulphoxide protects mainly the cell membrane, which would be sufficient to prevent loss of haemoglobin. In the experiments above, although few spermatozoa were motile after freezing and thawing in dimethyl sulphoxide, its protection afforded to the cell membrane, as measured by the percentage of cells not

penetrated by congo red stain after thawing, is quite good, 8% (v/v) dimethyl sulphoxide protecting the cell membrane as well as 7% (v/v) glycerol. Dimethyl sulphoxide apparently can protect more cell components than the membrane, since mouse bone marrow cells frozen in 15% (v/v) dimethyl sulphoxide will, upon injection, prevent death of lethally irradiated animals (Ashwood-Smith 1961*b*) better than cells frozen in 15% (v/v) glycerol. However, Richards and Persidsky (1961) found that bone marrow survived freezing better in 10 than 15% glycerol.

TABLE 7

SUMMARY OF ANALYSES OF VARIANCE OF THE DATA IN EXPERIMENT 5

Source of Variation	Degrees of Freedom	Variance Ratios				
		Motility Score		Percentage Motile		Percentage Unstained
		Thawed	Incubated	Thawed	Incubated	Thawed
Glycerol (*A*)	2					
3·0% *v.* 7·0% (*L*)	1	2·06	0·16	14·87***	4·48*	15·19***
5·0% *v.* mean of 3·0 and 7·0% (*Q*)	1	3·74	1·73	4·64*	0·17	4·36
Dimethyl sulphoxide (*B*)	3					
Mean of 0 and 1·5% *v.* mean of 3·0 and 4·5% (*L*)	1	7·81*	1·50	7·38*	1·02	6·84*
Mean of 1·5 and 3·0% *v.* mean of 0 and 4·5% (*Q*)	1	3·81	2·03	2·29	1·22	3·58
Remainder	1	3·64	0·54	2·16	0·33	0·34
Ejaculates (*C*)	3	3·15	1·39	0·08	1·91	7·51**
Interactions						
$A \times B$	6					
$L \times L$	1	1·65	0·03	4·46*	0·02	0·69
$L \times Q$	1	2·93	0·29	7·22*	2·03	0·03
$Q \times Q$	1	0·55	0·53	3·40	2·03	1·13
Remainder	3	0·42	0·36	1·24	0·39	0·09
$A \times C$	6	3·31	0·83	1·39	0·50	0·94
$B \times C$	9	1·07	1·12	2·99	1·03	0·29
Residual mean square	18	0·55	1·74	51·15	39·27	25·34

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

Jones (1965) found that during incubation at 5°C, dimethyl sulphoxide was much more toxic to spermatozoa than glycerol so that the protective action of dimethyl sulphoxide during freezing could be masked by its toxicity to spermatozoa. Thus, in these studies, 6% (v/v) dimethyl sulphoxide gave the highest revival rate, whilst with bone marrow cells 10% (Persidsky and Richards 1963) and 15% (Ashwood-Smith 1961*b*) was used, and with erythrocytes 10–15% was best (Lovelock and Bishop 1959). The short equilibration time (10 min) necessary before fr eezing bone marrow

cells in dimethyl sulphoxide (Persidsky and Richards 1963) may indicate a degree of toxicity to these cells. However, the combination of a very short equilibration in dimethyl sulphoxide and resuspension in a diluent free of dimethyl sulphoxide after thawing did not markedly increase revival in these expeirments. Such a process was indicated by the work of Martin (1963*a*) who found that, unless dog

TABLE 8

EXPERIMENT 6: THE EFFECTS OF FRUCTOSE, RECONSTITUTED MILK SOLIDS, GLYCEROL, TONICITY, AND OF THE PRESENCE OF GLYCEROL DURING STORAGE AT 5°C ON THE REVIVAL RATES AND SUBSEQUENT SURVIVAL DURING INCUBATION AT 37°C FOR 1 HR OF RAM SPERMATOZOA DEEP-FROZEN TO −79°C

Treatment			Mean Motility Score		Mean Percentage Motile		Mean Percentage Unstained
			Thawed	Incubated	Thawed	Incubated	Thawed
Final diluent composition (*A*) and tonicity (*B*)							
Milk solids (% w/v)	Fructose (% w/v)	Tonicity					
8·0	0·55	1·0	2·34	1·56	24·4	14·7	49·8
8·0	1·65	1·2	2·75	2·06	33·1	19·4	54·8
8·0	2·75	1·4	2·66	2·38	30·0	25·0	52·7
7·0	1·25	1·0	2·38	1·56	21·9	14·1	47·9
9·0	1·25	1·2	2·69	2·19	35·0	23·8	54·1
10·5	1·25	1·4	2·81	2·28	35·6	23·1	56·8
Glycerol (*C*)							
4% (v/v)			2·47	1·90	25·2	18·7	57·5
8% (v/v)			2·74	2·11	34·8	21·4	47·9
Storage at 5°C (*D*)							
Equilibrated 5 hr			2·68	1·92	29·6	18·3	51·0
Aged 5 hr			2·53	2·09	30·4	21·7	54·4
Ejaculates (*E*)							
1			2·06	1·54	23·3	13·5	62·1
2			2·52	1·67	25·4	13·5	48·1
3			2·83	2·23	27·9	22·1	43·0
4			3·00	2·58	43·3	30·8	57·5

spermatozoa were resuspended in a medium free of glycerol immediately upon thawing, poor activity was observed. In these experiments, little information was gained by resuspending thawed spermatozoa in a diluent free of the protective agent and incubating at 37°C for 1 hr. The difference between the responses measured decreased and those that were significant immediately after thawing were not significant after incubation for 1 hr.

In all of the experiments where the concentration of glycerol was increased in the freezing diluent, e.g., from 3 to 6–7%, scores of motility and percentage motile

spermatozoa on thawing also increased, but a greater proportion of spermatozoa were penetrated by congo red stain. A similar reversal of response was observed when spermatozoa were frozen in dimethyl sulphoxide.

The improvement following the addition of 1·5% (v/v) dimethyl sulphoxide to a diluent containing 7% glycerol is large enough to warrant further investigation. However, other factors, e.g. evaluation of other protective agents described in the literature, processing times, and diluent composition may produce equal or greater improvements in the freezing technique.

TABLE 9

SUMMARY OF ANALYSES OF VARIANCE OF THE DATA IN EXPERIMENT 6

Source of Variation	Degrees of Freedom	Variance Ratios				
		Motility Score		Percentage Motile		Percentage Unstained
		Thawed	Incubated	Thawed	Incubated	Thawed
Relative tonicity (*A*)	2					
Tonicity 1·2 *v.* 1·4	1	0·02	1·38	0·18	1·03	0·03
Tonicity 1·0 *v.* mean of tonicities 1·2 and 1·4	1	13·70***	19·60***	23·12***	15·93***	7·32**
Cause of tonicity (*B*) (fructose *v.* milk solids)	1	0·20	0·00	0·27	0·57	0·01
Glycerol level (*C*)	1	8·36**	2·39	19·76***	1·26	24·68***
Storage at 5°C (*D*)	1	2·42	1·57	0·19	2·54	3·12
Ejaculates (*E*)	3	19·35***	11·90***	16·06***	13·35***	22·08***
Interactions						
Pooled treatment × treatment interactions	13	1·74	0·26	0·42	0·81	0·83
Pooled treatment × ejaculate interactions	21	1·36	2·04	0·90	1·12	0·87
Residual mean square	53	0·84	1·92	49·35	68·30	30·77

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

Blackshaw (1960) froze semen in various concentrations of reconstituted skim milk containing 1·25% (w/v) fructose. The semen was cooled to 5°C in 7, 9, and 11% (w/v) of milk solids, but since glycerol replaced some of the milk the final levels in his diluents were: 6·5, 8·3, and 10·2% (w/v) milk solids when 7·5% glycerol was used. Spermatozoa revived best when frozen in the highest concentration of milk. In experiment 6, tonicity proved to be the important factor, rather than the relative levels of fructose and milk contributing to tonicity. Further studies are necessary to determine the relative importance of the protein, lactose, or electrolyte fraction of milk in diluents for deep-freezing spermatozoa.

Blackshaw (1960) in one experiment found it advantageous to equilibrate ram spermatozoa for 18 hr before freezing, but in a second experiment equilibration had

no effect. First, Henneman, and Magee (1959) found no difference between equilibration times of 1 and 18 hr. With bull semen, Martin (1961) found that storage for 6 hr at 5°C in the presence or absence of glycerol is required before freezing. Other workers do not agree that equilibration for 6 hr is enough (Cragle and Myers 1954; Miller and Van Demark 1954; Saroff and Mixner 1955). In experiment 3, equilibration for 5 hr decreased the revival of two ejaculates frozen in glycerol but had no effect on the other two ejaculates. Experiment 6 demonstrates that spermatozoa survive equally well after storage at 5°C either in the presence or absence of glycerol. This is in agreement with results obtained with bull semen (Martin 1963*b*). With this species, processing time from collection until the time glycerol is added may affect the period of equilibration necessary (Polge 1957); however, Martin's (1963*b*) results do not indicate this. In these researches, providing spermatozoa are stored at 5°C for some hours before adding glycerol, an equilibration time as short as 10 min is satisfactory. Although the variability in response to equilibration in glycerol may be attributed to ejaculate differences, an as yet unnamed factor may be causative.

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