INFLUENCE OF EQUILIBRATION, FREEZING RATE, METHOD OF DILUTION, AND DILUENT ON THE SURVIVAL OF DEEP-FROZEN BULL SPERMATOZOA

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Summary

From the results of four factorial experiments on the deep-freezing of bull spermatozoa:

(1) Revival rates of semen treated with lecithin followed by cooling to 5°C with dilution just before freezing did not differ significantly from samples diluted at 30°C soon after collection of the ejaculate. Aging the spermatozoa at 5°C for 6 hr before freezing was beneficial and a slow freezing rate of 0·5 to 1 degC fall per minute to −15°C followed by 3 degC fall below this temperature gave better results than faster rates. Time of storage at 5°C and freezing rate interacted, as fast freezing was much better tolerated by spermatozoa which had been aged at 5°C for 6 or 18 hr.

(2) The addition of glycerol independently to further dilution of the diluted samples held at 5°C had no effect on the aging or equilibration effect. Eosin and congo red used as supra-vital stains gave substantially the same results, showing an increase in the percentage of unstained spermatozoa as the storage period at 5°C was increased from 2 to 18 hr.

(3) Equilibration for 8 hr was better than for 2 or 18 hr. Rapid freezing was detrimental, and motility scores on thawing fell in a period of storage at −79°C of 3 months.

(4) Diluted semen was stored for 2, 4, 6, and 8 hr at 5°C and the glycerol-containing solution was added at the beginning or end of this period or half way through it. The effect of the inclusion of 1·25% (w/v) fructose in the diluent for freezing was also studied. On thawing, the best revival rates were observed in samples stored for 8 hr and glycerol added after half the storage period had elapsed. Incubation for 2 hr at 37°C after centrifugation of the samples and resuspension of the spermatozoa in a diluent free of glycerol and containing 0·1% fructose showed that longevity after thawing was improved significantly by storage for 8 hr before freezing and the inclusion of fructose in the diluent for freezing.

I. INTRODUCTION

Polge (1957) reviewed the various reports on the importance of equilibrating spermatozoa with the glycerol-containing diluent for some hours at 5°C before deep-freezing and, although unable to offer an explanation of the mechanism involved which gave improvement in revival rates, presented further information to show that spermatozoa tolerated the damaging effects occurring between −15 and −25°C

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during deep-freezing better after equilibration for 18 hr. An investigation of the equilibration effect using bull spermatozoa (Martin 1963*a*) showed that an improvement in survival after deep-freezing occurred if the diluted semen was stored at 5°C for up to 12 hr after dilution. However, this was not dependent on the presence of glycerol in the diluent during the chilled storage. Further studies (Martin 1963*b*, 1963*c*) showed that the effect occurred in a variety of diluents and that the inclusion of fructose in the diluent improved revival rates but did not eliminate the desirability of a period of storage at 5°C before freezing.

TABLE 1

DILUENTS USED IN THE EXPERIMENTS: SEMEN WAS DILUTED IN THE FIRST DILUENT AT 30°C AND FURTHER DILUTED IN THE DILUENTS CONTAINING GLYCEROL AT 5°C

	Egg Yolk (% v/v)	Lecithin (% w/v)	Sodium Citrate (mM)	Phosphate Buffer (mM) (pH = 7·0)	Fructose (mM)	Glycerol (M)
Experiments 1, 2, and 3						
First stage	—	1	60	20	60	—
Second stage	—	1	60	20	60	2*
Final composition	—	1	60	20	60	1
Experiment 4						
First stage	50	—	40	10	—	—
Second stage						
No added fructose	—	—	80	20	—	2
With added fructose	—	—	80	20	140	2
Final composition	25	—	60	15	0 or 70	1

* 1, 6, and 10M solutions of glycerol were also prepared for treatments 1 and 4 of experiment 2 (see Table 4).

Studies of rates of cooling of bull spermatozoa have shown that cooling during deep-freezing must be slow initially but higher rates are possible below −10°C. Bruemmer, Eddy, and Duryea (1963) found that rates lying within 1 and 5 degC fall per minute to −10°C and within 4–20 degC per minute between −10 and −30°C gave the highest survival rates of spermatozoa. This recent work demonstrated the existence of a range of satisfactory cooling rates within which the results of other workers (Polge 1953; Erickson, Graham, and Frederick 1954; Van Demark and Kinney 1954; Martin 1962) fell. Polge (1957) and Polge and Jakobsen (1959) showed that the degree of damage occurring between −15 and −25°C during deep-freezing was related to equilibration time, best revival rates following an equilibration period of up to 8 hr when fast or slow cooling rates were equally satisfactory. Survival of storage at −79°C for a period of months was also thought to be related to these factors.

In this paper the factors of equilibration, freezing rate, and storage time at −79°C are investigated together with the effects of the inclusion of fructose in the diluent and variations in the timing and method of addition of glycerol to the diluted semen.

II. Materials and Methods

Bull semen was collected with an artificial vagina, the ejaculates were held at 30°C, and the initial dilution was made at this temperature within 30 min of collection. The diluents used are listed in Table 1. Diluted semen was cooled from 30 to 5°C in 2 hr. To avoid temperature shock of the semen cooled after minimum dilution in experiment 1, each 1 ml of semen was treated with 0·03 ml of a 30% w/v emulsion of

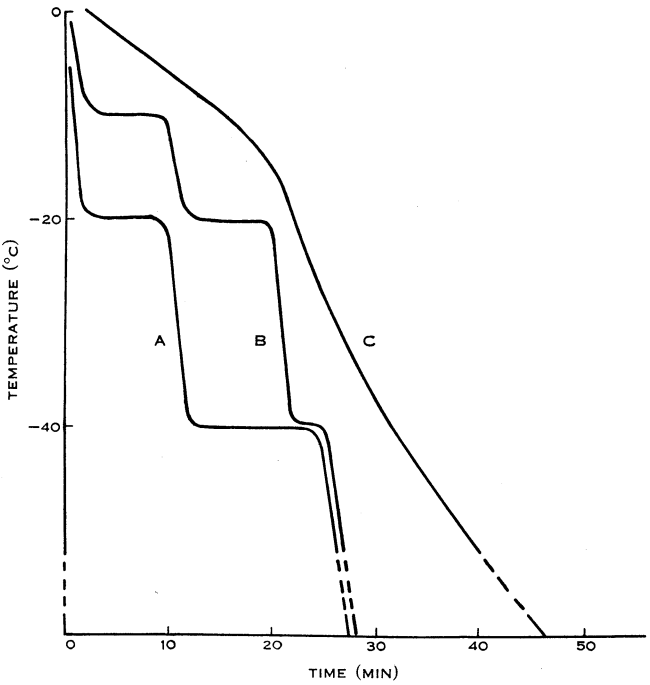

Fig. 1.—Freezing rates used in the experiments. Rates A and B were given by transferring the ampoules of semen from a water-bath at 5°C to alcohol-baths at −10, −20, and −40°C for the times shown. A container patterned on that described by Polge and Loveleck (1952) gave rate C.

egg lecithin (Blackshaw 1954; Martin 1963*a*) in buffered citrate. For every treatment combination requiring it, 0·03 ml of this semen was pipetted into 0·97 ml of final diluent in an ampoule, giving 1 ml of diluted semen ready for immediate freezing. Semen for the control treatments was diluted and cooled in the lecithin-containing, buffered citrate–fructose diluent. After the required period of storage, for each ampoule of diluted semen required, 0·05 ml of chilled semen was mixed with 0·5 ml of diluent containing glycerol, giving a final preparation matching the other in dilution rate and diluent composition.

All semen samples for experiment 2 were first diluted 20-fold. When at 5°C, four methods were used to complete dilution to 40-fold as is shown in Table 4.

Freezing rates.—For experiment 1, rates A, B, and C (Fig. 1) were used. For rate A, 1 ml ampoules of semen were transferred from 5°C to an alcohol-bath at −20°C for 10 min and then cooled from −20 to −40°C by transfer to a bath at −40°C where they remained for 15 min before being packed in crushed dry-ice for storage at −79°C. Rate B was given by first holding the ampoules in a bath at −10°C for 10 min and transferring them to the bath at −20°C for 10 min and then into the bath at −40°C for 5 min before packing in dry-ice. Samples were cooled at rate C by using a freezing container patterned on that of Polge and Lovelock (1952).

TABLE 2

MEAN REVIVAL RATES FOR BULL SPERMATOZOA FROZEN AFTER VARIOUS PERIODS OF STORAGE AT 5°C (EXPERIMENT 1)

Treatment	Motility	No. of Spermatozoa Motile (%)	No. of Spermatozoa Unstained (%)
Period of storage at 5°C (*A*):			
0 hr	2·39	21·7	16·7
6 hr	2·87	29·8	30·2
18 hr	2·50	24·8	35·5
Freezing rate (*B*):			
Method A } See Figure 1	2·03	15·9	17·7
Method B } See Figure 1	2·68	26·7	29·3
Method C } See Figure 1	3·06	33·7	35·3
Stage of experiment when semen diluted (*C*):			
At the start of the experiment	2·60	24·9	32·3
Immediately before freezing	2·58	26·0	22·6
Ejaculates (replicates) (*D*):			
1	2·29	30·0	35·7
2	2·35	28·1	39·8
3	2·86	24·2	19·0
4	2·85	19·4	15·3

All samples of semen were stored deep-frozen at −79°C for at least 24 hr before thawing. Ampoules of deep-frozen semen were thawed rapidly in a water-bath at 40°C and scores of revival were made immediately after. Additionally, in experiment 4, 1-ml samples from every treatment combination were centrifuged at 300 *g* for 10 min, the supernatant removed, and the spermatozoa resuspended in 0·8 ml of buffered citrate (80 mM sodium citrate, 20 mM phosphate buffer, 4 mM potassium chloride, and 6 mM fructose). These samples were then incubated for 2 hr at 37°C and further scores of viability were made. Motility, percentage motile, and percentage unstained by congo red (3% w/v) or eosin (0·6% w/v)–nigrosin (5% w/v) mixture were measured as described previously (Martin 1963*a*).

The experiments were factorial in design: experiment 1, $2\times3^2\times4$; experiment 2, 3×4^2; experiment 3, 2×3^3; and experiment 4, $2\times3^2\times4$. The SILLIAC computer was used for the analyses of variance and covariance. In these analyses, the terms

"linear" (L) and "quadratic" (Q) and the corresponding partition of interaction variance using orthogonal polynomials are described by Cochran and Cox (1957). The other set of orthogonal coefficients used in experiment 2 is shown in Table 4.

III. Results

Table 2 gives the mean revival of deep-frozen spermatozoa after storage for 0, 6, or 18 hr at 5°C before deep-freezing and following three rates of freezing. The analyses of variance are summarized in Table 3. On scores of activity, storage for 6 hr

Table 3

Summary of analyses of variance of the data of experiment 1

Source of Variation	Degrees of Freedom	Variance Ratios		
		Motility	Percentage Motile Spermatozoa	Percentage Unstained Spermatozoa
Period at 5°C (A)				
0 *v.* 18 hr (L)	1	0·72	6·48*	106·11***
6 *v.* 0 and 18 hr (Q)	1	16·11***	13·63***	6·58*
Freezing rate (B)				
A *v.* C (L)	1	70·17***	91·41***	98·47***
B *v.* A and C (Q)	1	1·72	3·47	6·03*
Time of dilution of semen (C)	1	0·61	0·81	34·69***
Ejaculates (D)	3	9·57***	8·68***	59·75***
Interactions				
$A \times B$				
$L \times L$	1	11·04**	17·17***	4·67*
$L \times Q$	1	8·01**	15·78***	3·02
Remainder	2	1·82	1·50	0·74
$A \times C$				
$L \times L$	1	6·82*	0·05	2·79
$Q \times L$	1	0·27	0·14	2·28
$B \times C$	2	0·44	0·37	0·50
$A \times D$	6	1·51	2·46*	1·24
$B \times D$	6	1·61	1·03	0·72
$C \times D$	3	1·44	0·66	1·37
Residual	40	0·181†	21·6†	21·0†

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

was superior to the longer or shorter period, but the percentage of unstained spermatozoa was highest if storage for 18 hr was allowed. Freezing rate C was better than the faster rates. There was a significant overall reduction of numbers of unstained spermatozoa if dilution was delayed until immediately before the start of freezing.

The rate of freezing interacted with time of storage at 5°C and Figure 2 shows that there was a very poor percentage of motile spermatozoa after rapid freezing following no storage period at 5°C. Storage at 5°C for 6 or 18 hr made the spermatozoa better able to withstand freezing and similar results were found for the percentage of

unstained spermatozoa. The interaction, found only in the data on motility score, of dilution method and time of storage was not a major factor, showing that complete dilution just before freezing gave somewhat better motility than the usual process when there was no period of storage with a reverse effect after 18 hr. This contrast within the interaction did not involve results from samples frozen after storage for 6 hr which was noted above as superior to the use of other times of storage before freezing.

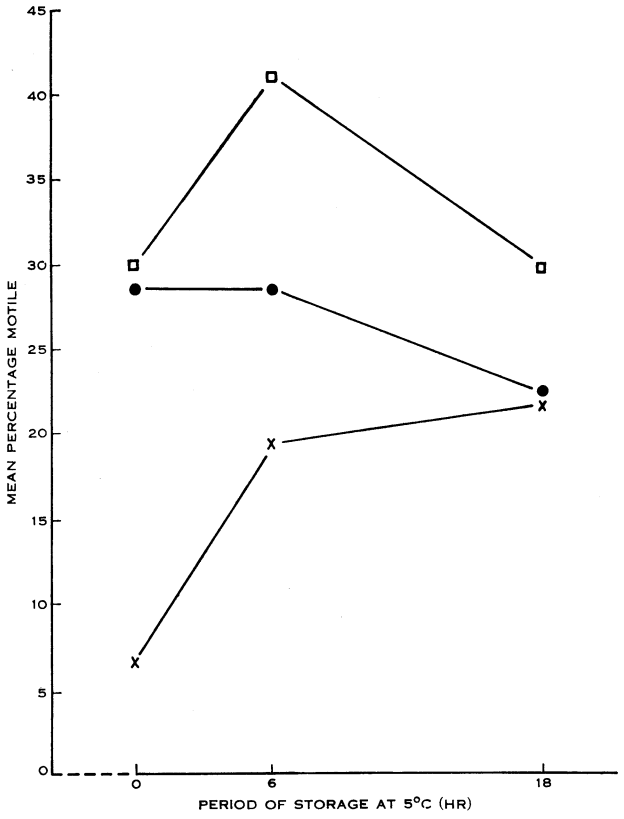

Fig. 2.—Effect of storage time at 5°C before freezing on the percentage of motile spermatozoa after deep-freezing. × Rapid freezing (rate A). ● Intermediate freezing (rate B). □ Slow freezing (rate C).

The mean results of experiment 2 are summarized in Table 4. The variations in glycerol addition and dilution procedures did not have any great effect. The addition of an equal volume of a 15% (2·0M) glycerol solution to the diluted semen as soon as it was at 5°C made it just significantly superior in motility and percentage unstained by eosin to the semen in which the dilution rate was increased as soon as the samples reached 5°C but the glycerol addition was delayed until just before deep-freezing (contrast *b*, Tables 4 and 5). The percentage of unstained spermatozoa rose as the storage period was increased from 2 to 18 hr, but this reached significance only in the counts from eosin-stained smears. There was, however, quite close similarity in the means for counts made for both eosin and congo red.

The interaction of dilution method and storage time at 5°C ($A \times B$) was just significant and indicated that the motility of spermatozoa was lowest when they were frozen after 18 hr of storage using dilution method 4. There was a similar, but not significant trend in the scores of percentage of motile spermatozoa.

TABLE 4

EFFECTS OF FOUR PROCESSING METHODS BEFORE FREEZING ON THE REVIVAL OF DEEP-FROZEN BULL SPERMATOZOA (EXPERIMENT 2)

Method	Volume of Diluent (ml) Added to each 0·5 ml of Diluted, Chilled Semen		Molarity of Glycerol in Diluted Semen during Storage at 5°C	Coefficients Used for Contrasts in Analysis of Variance (Table 5)		
	Added as soon as Samples had Cooled to 5°C	Added Immediately before Freezing		*a*	*b*	*c*
1	0·1 (6M)*	0·4 (1M)	1	+1	0	+1
2	0·5 (2M)	—	1	0	+1	−1
3	—	0·5 (2M)	0	−1	0	+1
4	0·4	0·1 (10M)	0	0	−1	−1

Treatment	Mean Scores of Revival			
	Motility	Percentage Motile	Percentage Unstained	
			Congo Red	Eosin
Dilution and glycerol addition				
Method 1 (as above)	2·94	27·1	27·6	28·9
Method 2	3·16	32·5	34·7	35·1
Method 3	3·07	28·3	33·5	34·1
Method 4	2·93	27·5	33·6	29·7
Storage period at 5°C before deep-freezing (*B*):				
2 hr	3·06	30·3	31·4	29·0
6 hr	3·02	29·4	30·0	29·9
18 hr	2·99	26·9	35·7	36·9
Ejaculates (replicates) (*C*):				
1	2·62	24·2	21·7	21·8
2	3·05	35·0	43·4	40·7
3	3·05	23·7	25·8	25·7
4	3·37	32·5	38·4	39·5

* Values in parentheses are molarities of glycerol in the diluents used at each stage.

Experiment 3 is summarized in Tables 6 and 7. On activity of spermatozoa after thawing, equilibration for 8 hr was best, but 8 and 18 hr were equivalent and considerably higher than the counts of unstained spermatozoa from samples equilibrated for 2 hr. The rapid freezing rate (method A) depressed revival significantly.

TABLE 5

SUMMARY OF ANALYSES OF VARIANCE OF THE DATA OF EXPERIMENT 2

Source of Variation	Degrees of Freedom	Variance Ratios			
		Motility	Percentage Motile Spermatozoa	Percentage Unstained Spermatozoa	
				Congo Red	Eosin
Dilution method (A)					
Coefficient a } (see Table 4)	1	1·56	0·28	3·48	3·50
Coefficient b } (see Table 4)	1	5·69*	4·37	0·31	5·33*
Coefficient c } (see Table 4)	1	0·34	2·02	3·56	0·44
Storage time at 5°C (B)	2	0·34	0·83	2·33	7·94**
Ejaculates (C)	3	20·00***	11·32***	21·18***	27·22***
Interactions					
$A \times B$	6	3·10*	0·97	0·47	1·83
$A \times C$	9	1·90	3·05*	1·42	3·33*
$B \times C$	6	2·25	4·51**	1·50	4·78**
Residual	18	0·058†	14·4†	25·3†	18·0†

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

TABLE 6

EFFECT OF EQUILIBRATION TIME, FREEZING RATE, AND PERIOD OF STORAGE AT −79°C ON DEEP-FROZEN BULL SPERMATOZOA (EXPERIMENT 3)

Treatment	Mean Scores of Revival		
	Motility	No. of Spermatozoa Motile (%)	No. of Spermatozoa Unstained (%)
Equilibration time:			
2 hr	2·38	21·1	32·9
8 hr	2·59	22·8	47·9
18 hr	2·21	21·2	47·2
Freezing rate			
Fast (A, Fig. 1)	2·24	19·7	37·2
Normal (C, Fig. 1)	2·55	23·7	48·1
Storage period at −79°C:			
1 day	2·56	23·3	42·3
1 month	2·47	21·4	44·3
3 months	2·15	20·4	41·4
Ejaculates (replicates):			
1	3·13	28·6	52·7
2	1·56	11·2	34·8
3	2·47	25·3	40·6

There was a small but significant decline in motility of spermatozoa thawed after storage at −79°C for 3 months.

Fast freezing was better tolerated as scored by percentage unstained by congo red after an equilibration period of 8 hr (interaction $A \times B$) although results were still below those for slow freezing. Thus 25·3 and 40·6% of spermatozoa were unstained by congo red after fast and slow freezing respectively when frozen after 2 hr equilibration whereas after 18 hr equilibration the equivalent results were 44·2 and 50·2%. Differences in degree of response to the treatments accounted for the significant ejaculate × treatment interactions.

TABLE 7

SUMMARY OF ANALYSES OF VARIANCE OF THE DATA OF EXPERIMENT 3

Source of Variation	Degrees of Freedom	Variance Ratios		
		Motility	Percentage Motile Spermatozoa	Percentage Unstained Spermatozoa
Equilibration (A)				
2 hr *v.* 18 hr (L)	1	1·69	1·08	42·29***
8 hr *v.* 2 and 18 hr (Q)	1	6·38*	3·01	15·54***
Freezing rate (B)	1	8·37**	6·15*	34·16***
Storage time at −79°C (C)				
1 day *v.* 3 months (L)	1	9·50**	3·78	0·06
Remainder (Q)	1	1·00	0	1·38
Ejaculates (D)	2	70·87***	61·57***	31·77***
Interactions				
$A \times B$				
$L \times L$	1	1·00	0·10	5·06*
$Q \times L$	1	1·62	0·20	0·06
$A \times C$	4	0·69	1·23	0·89
$B \times C$	2	0·75	0·32	1·38
$A \times D$	4	3·75*	7·79***	1·19
$B \times D$	2	0·44	0·16	1·45
$C \times D$	4	3·81*	1·51	1·23
Residual	28	0·16†	15·6†	16·6†

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

In experiment 4 spermatozoa were frozen slowly (rate C) after a period of storage at 5°C of 2, 4, 6, or 8 hr, either in a diluent having no added fructose or in one containing 1·25% (w/v) fructose. In preparation for freezing at each one of these times, glycerol was added as soon as the diluted semen had cooled to 5°C [equilibrated, treatment (*a*), Table 8], after half the storage period had elapsed, i.e. 1, 2, 3, and 4 hr equilibration respectively [treatment (*b*), Table 8], or just before freezing started [treatment (*c*)]. Scores made immediately after thawing showed that revival was significantly improved by a storage period of 8 hr and that, on motility and percentage unstained, the mean effect of equilibration treatment (*b*) was superior to the others (Table 9).

The addition of fructose to the diluent in which the spermatozoa were frozen had no effect on their activity on thawing but the percentage of unstained spermatozoa was significantly raised.

Samples from ejaculate 2 were severely damaged by deep-freezing after only storage for 2 hr at 5°C and also the percentage of unstained spermatozoa in ejaculate 1

TABLE 8

EFFECTS OF EQUILIBRATION TIME AND OF FRUCTOSE IN THE DILUENT ON DEEP-FROZEN BULL SPERMATOZOA (EXPERIMENT 4)

Scores were made immediately after thawing and after incubation at 37°C for 2 hr

Treatment	Mean Scores of Revival and Survival					
	Motility		Percentage Motile Spermatozoa		Percentage Unstained Spermatozoa	
	On Thawing	After Incubation	On Thawing	After Incubation	On Thawing	After Incubation
Period of storage at 5°C before deep-freezing (*A*):						
2 hr	2·50	1·29	21·4	6·2	26·8	21·0
4 hr	2·84	2·14	31·1	16·9	40·8	31·0
6 hr	2·91	2·12	33·6	20·6	43·3	33·3
8 hr	2·99	2·44	38·9	24·2	46·0	35·9
Ratio of equilibration period to total storage period at 5°C (*B*)						
(*a*) Glycerol added as soon as semen had cooled to 5°C	2·81	2·08	31·9	17·2	39·6	30·5
(*b*) Glycerol added after half of storage period had elapsed	2·96	2·01	32·9	18·9	42·5	31·8
(*c*) Glycerol added immediately before deep-freezing	2·67	1·91	28·9	14·9	35·5	28·7
Fructose content of diluent (*C*):						
0	2·76	1·68	29·4	12·7	35·8	26·5
1·25 (% w/v)	2·86	2·32	33·0	21·3	42·7	34·2
Ejaculates (*D*):						
1	3·31	2·96	40·8	31·5	46·5	35·3
2	2·23	1·23	24·4	7·3	38·1	28·1
3	2·89	1·85	28·5	12·1	33·2	27·6

was not markedly improved by the addition of fructose to the diluent. These variations in response to treatment account for the significant treatment × ejaculate interactions in Table 9.

Analysis of the scores made after incubation of the thawed samples showed that storage at 5°C for 8 hr before freezing and the use of fructose in the diluent for freezing were highly beneficial, but the time of addition of glycerol before freezing had no significant effect on continued survival after thawing (Table 10). The percentage of motile spermatozoa surviving incubation after freezing showed that adding fructose

to the diluent before freezing had a highly significant effect on all scores of survival of incubation after allowing for the effects observed immediately after thawing by using the scores made at thawing as a covariate (Fig. 3).

Spermatozoa from ejaculate 2 did not survive incubation as well as those from the other ejaculates and this was particularly marked where samples had been frozen after storage at 5°C for 2 hr. This gave the significant $A \times D$ interaction in Table 10.

TABLE 9

SUMMARY OF ANALYSES OF VARIANCE OF REVIVAL RATES IN EXPERIMENT 4

Source of Variation	Degrees of Freedom	Variance Ratios		
		Motility	Percentage Motile Spermatozoa	Percentage Unstained Spermatozoa
Storage period at 5°C (A)				
Linear regression (L)	1	16·00***	46·87***	76·98***
Quadratic curvature (Q)	1	2·11	2·20	14·84***
Remainder (C)	1	0·50	1·51	2·52
Ratio of equilibration to total storage period (B)				
(a) *v.* (c) (L)	1	1·81	1·28	4·50*
(b) *v.* (a) and (c) (Q)	1	6·08*	2·09	10·30**
Fructose (C)	1	1·28	3·89	18·74***
Ejaculates (D)	2	53·30***	30·39***	25·58***
Interactions				
$A \times B$	6	2·26	1·70	0·65
$A \times C$	3	0·54	0·81	0·79
$B \times C$	2	0·66	0·23	1·37
$A \times D$	6	2·50*	2·41*	1·58
$B \times D$	4	1·41	2·06	2·70*
$C \times D$	2	0·56	0·76	7·98**
Residual	40	0·133†	23·44†	15·57†

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

IV. DISCUSSION

In the first experiment storage for 6 hr at 5°C before freezing was shown to give the best mean results. This occurred whether the semen was diluted before or after chilling to 5°C. In addition the improvement in the counts of unstained spermatozoa, regardless of the time of dilution, indicated that the effects of a period of storage at 5°C were not primarily related to the alteration in diluent from seminal plasma to buffered citrate. However, initial dilution at 30°C was the superior method of preparation.

Both scores of activity and the percentages of unstained spermatozoa showed that survival of rapid freezing increased in experiments 1 and 2 as the period of storage at 5°C was lengthened. Although, after any time of storage, fast-freezing was not as successful as the rate proposed by Polge and Lovelock (1952), this evidence confirms

the findings of Polge (1957), indicating the need to study freezing rates in conjunction with alterations in processing techniques and diluents in research for an optimal deep-freezing method. Both the rate of cooling from 30 to 5°C and the presence of fructose in the diluent have been shown to influence the revival rate after deep-freezing (Martin 1965). It seems possible that all these factors are associated with temperature shock and consequent damage to the cell membrane (Walton 1957) and a redistribution of ions between spermatozoa and diluent after changes in permeability of the spermatozoa (Blackshaw and Salisbury 1957) but any such changes in the spermatozoa of benefit to survival through the deep-freezing process remain unknown.

TABLE 10

SUMMARY OF ANALYSES OF COVARIANCE OF THE SCORES OF SURVIVAL AFTER INCUBATION IN EXPERIMENT 4, USING THE DATA COLLECTED IMMEDIATELY ON THAWING AS COVARIATE

Source of Variation	Degrees of Freedom	Variance Ratios		
		Motility	Percentage Motile Spermatozoa	Percentage Unstained Spermatozoa
Storage period at 5°C (*A*)				
2 *v.* 8 hr (*L*)	1	13·97***	31·97***	10·23**
Remainder	2	2·54	2·04	0·32
Ratio of equilibration to total storage period (*B*)	2	0·41	0·54	0·01
Fructose (*C*)	1	13·94***	29·27***	12·80**
Ejaculates (*D*)	2	22·97***	39·91***	1·41
Interactions				
$A \times B$	6	0·86	2·25	1·14
$A \times C$	3	1·11	2·30	1·98
$B \times C$	2	0·20	1·23	0·72
$A \times D$	6	1·32	3·51**	1·28
$B \times D$	4	0·57	1·03	1·00
$C \times D$	2	0·56	2·05	1·10
Residual	39†	0·519‡	36·72‡	12·71‡

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

† One degree of freedom subtracted in fitting covariance regression coefficient.

‡ Variance from reduced sums of squares.

Other contributory factors in the equilibration phenomenon could have been the response of the cell membrane to glycerol or to the increase in dilution rate when the glycerol-containing solution was added. Although some improvement in motility and percentage of spermatozoa unstained by eosin was shown when the diluted, chilled semen was equilibrated (i.e. as defined by Martin 1963*c*, the second dilution stage and glycerol addition were simultaneous) in experiment 2, revival rates were not greatly affected by the time at which glycerol was added or by the increase in dilution rate. The depression of revival in treatments 1 and 4 was probably more related to the difficulties of dispersing a high concentration of a viscid solution of glycerol before it damaged the spermatozoa it contacted. As essentially the same

results were given when spermatozoa were stained with eosin, the improvement in staining reaction after thawing associated with increased storage period at 5°C before freezing was not due to any special property of congo red.

A slight but significant decline in motility during storage at −79°C was found in the third experiment which is in agreement with Larson and Graham (1958), but there was no evidence of interactions of equilibration or freezing rate with storage time at −79°C. These could, however, exist if other diluents or periods of equilibration were used (Polge and Jakobsen 1959).

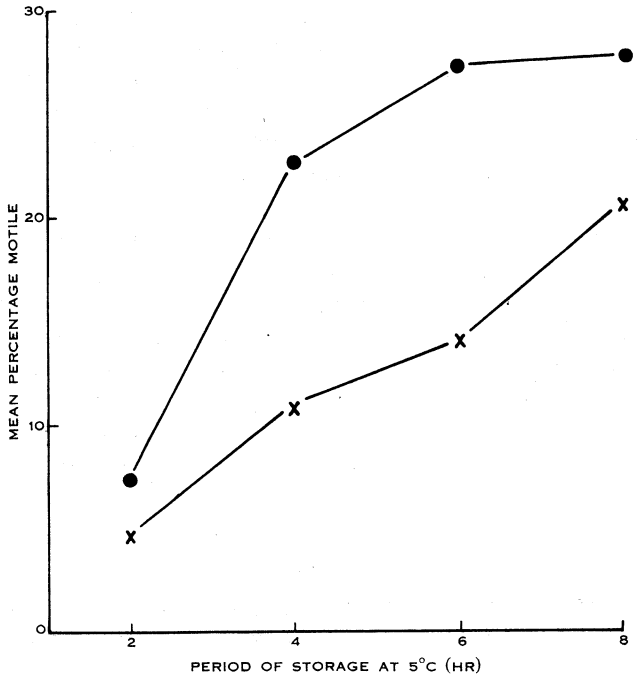

Fig. 3.—Survival of deep-frozen bull spermatozoa incubated for 2 hr at 37C° after thawing. × No fructose added to the glycerol-containing diluent for deep-freezing. ● 1·25% fructose added to the glycerol-containing diluent for deep-freezing.

All samples of deep-frozen semen in experiment 4 were centrifuged and resuspended in a standard buffered citrate diluent containing 1 mg/ml of fructose for incubation after thawing. Thus, samples containing no added fructose were resuspended in a diluent containing fructose, whereas those frozen in the diluent containing 1·25% could have, by the content of fructose remaining in the plug of spermatozoa after centrifugation, a somewhat higher level of fructose in the diluent for incubation. This final level is unlikely to have been higher than 1·4 mg/ml. The usual number of spermatozoa per millilitre of suspension for incubation was about 30×10^6 so that limitations of fructose availability in 2 hr incubation are not likely to have caused the very striking improvement in the survival rates after deep-freezing

in the presence of fructose. The addition of a relatively high concentration of fructose to the diluent for freezing appears to modify the physical properties of the diluent and favour the survival of bull spermatozoa.

From the four experiments reported in this paper and those published earlier (Martin 1963*a*, 1963*b*, 1963*c*, 1965), it is clear that the equilibration effect is variable in its magnitude, depending on the adequacy of the diluent and methods of processing. The introduction of a further test of viability, i.e. incubation at body temperature, has contributed fresh information and it is possible that much of the uncertainty about the importance of equilibration would have been removed by using this test in the earlier experiments. However, none of the designs used could show what changes occur in the spermatozoon, but they have demonstrated that, regardless of other variables, changes occur in this period of time which make the spermatozoa better able to withstand the stresses of freezing and thawing. The optimum freezing rate, the best equilibration period, or ratio of equilibration with glycerol to total storage time at 5°C and diluent have yet to be defined and it appears that, at some stage or other in the preparation of deep-frozen semen, these factors are interrelated. The period of storage at 5°C before freezing must be greater than 2 hr but it is unlikely that storage for longer than 12 hr is required, which is in agreement with Miller and Van Demark (1954), Cragle *et al.* (1955), Graham, Erickson, and Bayley (1957), Polge and Jakobsen (1959), and Sullivan and Mixner (1963). The use of tests of longevity after thawing as well as revival rates were shown to be valuable in the fourth experiment in this paper and this supports the evidence presented earlier (Martin and Emmens 1961) on the need for a period of storage at 5°C before freezing and the addition of fructose to the diluent for freezing when the fertility of deep-frozen semen is considered.

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