FREEZING RAM SPERMATOZOA BY THE PELLET METHOD

III.* THE EFFECTS OF PELLET VOLUME, COMPOSITION OF THE THAWING SOLUTION, AND RECONCENTRATION OF THE THAWED SEMEN ON SURVIVAL OF SPERMATOZOA

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Summary

Five factorial experiments were conducted to examine the effects of pellet volume, composition of the thawing solution, and reconcentration of the thawed semen on the survival of ram spermatozoa after deep-freezing by the pellet method.

The results showed that the volume of the pellet can be increased from 0·03 to 0·86 ml without reducing the post-thawing survival of the spermatozoa. There was no evidence of interactions between pellet volume and either diluent tonicity or glycerol concentration on survival.

Both the revival and survival of spermatozoa during post-thawing incubation were higher when the pellets were thawed in 44·4 mM glucose–80·6 mM citrate compared with 88·4 mM citrate solution. The results further indicated that post-thawing survival was improved by reducing the concentration of citrate in the sugar–citrate thawing solution to 72·8 mM, and for semen frozen in raffinose–yolk–citrate diluent, using lactose rather than glucose in the thawing solution.

Centrifugation of the thawed semen at 3000 r.p.m. for 15 min and removing up to 87·5% of the supernatant did not depress the *in vitro* survival of spermatozoa.

I. INTRODUCTION

The effects of diluent composition and preparative procedures on the post-thawing survival of ram spermatozoa frozen by the pellet method have been examined by Salamon (1968), Salamon and Lightfoot (1969), and Lightfoot and Salamon (1969). In these studies the semen was frozen as pellets of volume 0·03 ml, but observations with bull semen (Nagase and Niwa 1964; Peskovatskii and Habibullin 1968) indicate that larger pellets can be frozen without detrimental effect. A pellet of sufficient volume to provide the number of motile spermatozoa required for one insemination would facilitate both freezing and thawing procedures.

While semen frozen by conventional means is usually thawed simply by warming the container, semen frozen in pellet form necessitates the use of a thawing solution as otherwise revival is comparatively poor, particularly when low dilution rates are employed prior to freezing (Lightfoot and Salamon 1969). On the other hand, dilution of the semen both prior to freezing and at thawing reduces the concentration of motile spermatozoa much below that required to obtain fertility. For these reasons, in the experiments reported here, the effects of pellet volume, composition of the thawing solution, and post-thawing centrifugation were examined.

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II. Materials and Methods

(a) *Experimental Designs*

Five experiments were conducted and the design and treatment comparisons for each are presented in Section III.

(b) *General*

Semen for all experiments was collected from mature Merino rams by artificial vagina.

In each experiment two or more of the procedures associated with the freezing and thawing of semen were varied as described fully in Section III. When not varied the following standard procedures were adopted.

The semen was diluted (semen : diluent, 1 : 4 v/v) at 30°C with a single addition of a raffinose (166·5 mM)–sodium citrate (102 mM)–egg yolk (15% v/v) diluent to which was added 5% (v/v) glycerol. The diluted semen was then cooled to 5°C over a period of 1·5 hr and held at that temperature for an additional 3 hr prior to pellet freezing. A cooled Pasteur pipette was used for dropping the semen onto the dry ice. Pellet volumes greater than 0·030–0·033 ml (1 drop) were achieved by placing the required number of drops in rapid succession into enlarged cavities on the surface of a block of dry ice. When frozen, the pellets were stored in liquid nitrogen for a minimum of 48 hr.

The pellets were thawed (pellets : thawing solution, 1 : 3 v/v) in test tubes containing 80·6 mM sodium citrate–44·4 mM glucose solution held in a water-bath at 37°C.

The percentage of motile spermatozoa for each treatment was assessed under coverslip on a warm stage immediately after thawing, and reassessed at intervals of 2 hr during the subsequent incubation (8 hr, expt. 1; 6 hr, expts. 2, 3, and 4; 4 hr, expt. 5) at 37°C.

(c) *Statistical Analyses*

The data were examined by analysis of variance following angular transformation as described previously (Salamon and Lightfoot 1969).

III. Experimental and Results

(a) *Experiment 1*

The experiment was of factorial design (3 × 2 × 3) as shown below:

(1) Pellet volume: 0·03 *v.* 0·24 *v.* 0·45 ml.

(2) Dilution rate at thawing: 1 : 2 *v.* 1 : 4, pellets : thawing solution (v/v).

(3) Rams: two pooled ejaculates from each of three rams.

The mean values for the percentages of motile spermatozoa assessed during 8-hr post-thawing incubation for 0·03-, 0·24-, and 0·45-ml pellets (Table 1) were not significantly different. There was a significant interaction ($P < 0·01$) between

Table 1

Experiment 1: mean percentage of motile spermatozoa in relation to pellet volume, dilution rate at thawing, rams, and duration of incubation

Pellet Vol. (ml)	Motile Spermatozoa (%)	Dilution Rate at Thawing*	Motile Spermatozoa (%)	Rams	Motile Spermatozoa (%)	Duration of Incubation (hr)	Motile Spermatozoa (%)
0·03	34·7	1 : 2	36·9	1	35·6	0	42·2
0·24	37·2	1 : 4	36·3	2	36·5	2	39·3
0·45	38·0			3	37·8	4	37·6
						6	35·4
						8	28·9
P:	n.s.		n.s.		n.s.		< 0·001

* Pellets : thawing solution, v/v.

dilution rate at thawing and duration of incubation. The effect was due to the faster rate of decline in the percentage of motile spermatozoa towards the end of incubation, following initially higher values, for semen thawed at the higher dilution rate, as shown below:

Dilution Rate at Thawing (pellets : thawing solution, v/v)	Duration of Incubation (hr)				
	0	2	4	6	8
1 : 2	40·7	39·0	36·2	36·2	32·4
1 : 4	43·6	39·6	39·0	34·5	25·5

Means for the three rams were not significantly different.

(*b*) *Experiment 2*

The experiment was of factorial design ($3 \times 3 \times 3 \times 3$) as shown below:

(1) Level of sodium citrate in the diluent (raffinose, 166·5 mM): 34 *v.* 68 *v.* 102 mM.

(2) Glycerol concentration in the diluted semen: 2·4 *v.* 3·6 *v.* 4·8% (v/v).*

(3) Pellet volume: 0·032 *v.* 0·160 *v.* 0·800 ml.

(4) Rams: three pooled ejaculates from each of three rams.

* Corresponds to 3·0, 4·5, and 6·0% glycerol in the diluent.

Of the factors studied only glycerol concentration had a significant effect (linear, $P < 0{\cdot}05$) on the mean percentage of motile spermatozoa assessed during incubation (Table 2). Most variation was due to differences between individual rams, with means of 36·3, 38·4, and 39·5% motile spermatozoa ($P < 0{\cdot}001$). Although spermatozoa from the three rams varied in their tolerance to diluents of varying tonicity, best results in each case were obtained with the diluent containing 68 mM sodium citrate.

TABLE 2

EXPERIMENT 2: EFFECTS OF LEVEL OF SODIUM CITRATE AND GLYCEROL CONCENTRATION ON THE PERCENTAGE OF MOTILE SPERMATOZOA ASSESSED DURING POST-THAWING INCUBATION

Duration of Incubation (hr)	Sodium Citrate Level in the Diluent (mM)*			Glycerol Concentration in the Diluted Semen (% v/v)			Means
	34	68	102	2·4	3·6	4·8	
0	40·9	44·5	43·3	41·3	43·5	43·9	42·9
2	37·8	40·3	42·2	39·0	40·4	40·9	40·1
4	34·6	37·4	36·6	35·5	36·6	36·5	36·2
6	32·4	34·6	32·5	32·5	34·2	32·7	33·2
Means	36·4	39·2	38·6	37·1	38·7	38·5	38·0

* With 166·5 mM raffinose.

The effect of incubation on the percentage of motile spermatozoa was modified by interactions with level of sodium citrate ($P < 0{\cdot}01$; Table 2), glycerol concentration ($P < 0{\cdot}05$; Table 2), and pellet volume ($P < 0{\cdot}05$) but the effects in

each case were of small magnitude. Spermatozoa frozen in the largest pellets (0·8 ml) showed slightly better survival during incubation [Fig. 1(*a*)].

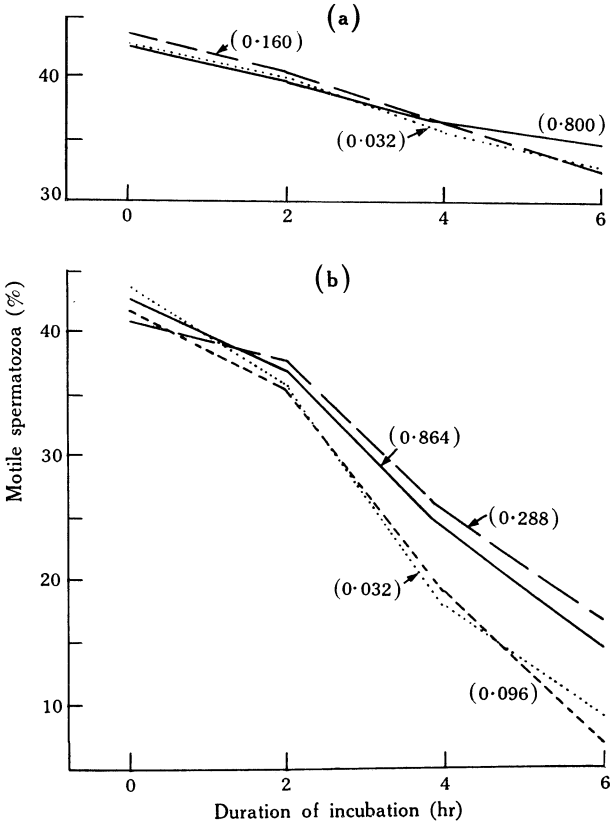

Fig. 1.—Effect of pellet volume on the survival of spermatozoa during the post-thawing incubation. Volume of pellet (ml) given in parenthesis.
(*a*) Experiment 2.
(*b*) Experiment 3.

(*c*) *Experiment 3*

The experiment was of factorial design (4 × 3 × 4) as shown below:

(1) Pellet volume: 0·032 *v.* 0·096 *v.* 0·288 *v.* 0·864 ml.

(2) Level of sodium citrate in the thawing solution (glucose 44·4 mM): 72·8 *v.* 80·6 *v.* 88·4 mM.

(3) Rams: three pooled ejaculates from each of four rams.

All three factors had statistically significant effects (Table 3) on the mean percentage of motile spermatozoa assessed during incubation. Results for the 0·032-, 0·096-, 0·288-, and 0·864-ml pellets were 25·2, 24·4, 30·1, and 29·3% respectively (linear, $P < 0·01$; cubic, $P < 0·05$). Although the percentages of motile spermatozoa, when assessed immediately after thawing, were similar for all four treatments, pellets of larger volume resulted in better spermatozoal viability during subsequent incubation [pellet volume × duration of incubation, $P < 0·001$; Fig. 1(*b*)].

The effect of incubation on the percentage of motile spermatozoa was also modified by an interaction with level of sodium citrate in the thawing solution ($P < 0·001$; Table 4). When examined immediately after thawing, poorest results

TABLE 3

EXPERIMENT 3: SUMMARY OF ANALYSIS OF VARIANCE

Source of Variation	D.F.	M.S.	Variance Ratio
Pellet volume (A)			
Linear	1		9·63**
Quadratic	1		0·00
Cubic	1		5·17*
Level of sodium citrate in the thawing solution (B)			
Linear	1		35·41***
Quadratic	1		3·06
Rams (C)	3		16·37***
Pooled (non-significant) first-order interactions	21		1·01
$A \times B \times C$ (error 1)	18	*32·414*	
Duration of incubation (D)			
Linear	1		155·15***
Quadratic	1		3·32
Cubic	1		1·59
$A \times D$			
Linear × linear	1		20·11***
Quadratic × linear	1		11·74**
Residual	7		0·48
$B \times D$			
Linear × linear	1		63·12***
Residual	5		1·16
$C \times D$†	9		5·82***
Pooled second-order interactions	63		0·86
$A \times B \times C \times D$ (error 2)	54	*13·156*	

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

† Component used to test the significance of duration of incubation (D).

TABLE 4

EXPERIMENT 3: EFFECT OF LEVEL OF SODIUM CITRATE IN THE THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA ASSESSED DURING POST-THAWING INCUBATION

Duration of Incubation (hr)	Sodium Citrate Level in Thawing Solution (mM)*			Means
	72·8	80·6	88·4	
0	39·6	44·8	42·2	42·2
2	39·0	37·7	33·0	36·5
4	29·7	22·9	14·6	22·1
6	18·0	13·4	5·7	11·8
Means	31·2	28·8	22·0	27·2

* With 44·4 mM glucose.

were obtained using the thawing solution of lowest tonicity (44·4 mM glucose, 72·8 mM sodium citrate). During subsequent incubation, however, spermatozoal survival was markedly superior in this solution.

TABLE 5

EXPERIMENT 4: SUMMARY OF ANALYSIS OF VARIANCE

Source of Variation	D.F.	M.S.	Variance Ratio
Sugar component of the diluent (*A*)			
Glucose *v.* lactose and raffinose	1		0·81
Lactose *v.* raffinose	1		1·53
Level of sodium citrate in the diluent (*B*)			
Linear	1		122·86***
Quadratic	1		49·98***
Rams (*C*)	3		5·67**
Sugar component of the thawing solution (*D*)			
Glucose *v.* lactose and raffinose	1		0·12
Lactose *v.* raffinose	1		5·96*
A × *B*			
Glucose *v.* lactose and raffinose × linear	1		44·56***
Glucose *v.* lactose and raffinose × quadratic	1		18·16***
Residual	2		0·70
A × D			
Lactose *v.* raffinose × lactose *v.* raffinose	1		7·98**
Residual	3		2·06
B × *C*†	6		3·46*
Pooled (non-significant) first-order interactions	16		1·37
Pooled second-order interactions	44		1·50
A × *B* × *C* × *D* (error 1)	24	*26·97*	
Duration of incubation (*E*)			
Linear	1		731·83***
Residual	2		0·85
A × *E*	6		1·80
B × *E*			
Linear × linear	1		309·92***
Quadratic × linear	1		44·92***
Residual	4		1·56
C × *E*	9		1·95
D × *E*			
Lactose *v.* raffinose × linear	1		12·44***
Residual	5		1·60
Pooled second-order interactions	90		1·43
Pooled third-order interactions	132		0·85
A × *B* × *C* × *D* × *E* (error 2)	72	*12·18*	

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

† Component used to test the significance of level of sodium citrate (*B*).

Spermatozoa from individual rams showed considerable variation in susceptibility to freezing and post-thawing incubation. Overall means for the four rams were 22·6, 23·3, 30·4, and 33·0 percentage motile spermatozoa ($P < 0·001$).

(*d*) *Experiment 4*

The experiment was of factorial design ($3 \times 3 \times 3 \times 4$) as shown below:
(1) Sugar component of the diluent: glucose *v.* lactose *v.* raffinose (166·5 mM).
(2) Level of sodium citrate in the diluent: 34 *v.* 102 *v.* 170 mM.
(3) Sugar component of the thawing solution (sodium citrate 80·6 mM): glucose *v.* lactose *v.* raffinose (44·4 mM).
(4) Rams: three pooled ejaculates from each of four rams.
The pellet volume was 0·03 ml.

There was a highly significant interaction between the sugar and level of sodium citrate in the diluent on the mean percentage of motile spermatozoa during incubation ($P < 0·001$: Table 5). The effect, shown in Table 6, was due to the comparatively better resistance of spermatozoa to high levels of sodium citrate when frozen in diluents containing glucose rather than lactose or raffinose.

TABLE 6

EXPERIMENT 4: EFFECT OF SUGAR COMPONENT OF THE DILUENT ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION AS MODIFIED BY INTERACTIONS WITH LEVEL OF SODIUM CITRATE IN THE DILUENT AND SUGAR COMPONENT OF THE THAWING SOLUTION

Sugar Component of Diluent (166·5 mM)	Sodium Citrate Level in Diluent (mM)			Sugar Component of Thawing Solution (44·4 mM)*			Means
	34	102	170	Glucose	Lactose	Raffinose	
Glucose	27·8	28·4	17·9	23·5	27·6	22·6	24·5
Lactose	32·4	34·5	10·3	25·3	23·2	25·5	24·7
Raffinose	33·6	34·2	12·1	25·7	28·0	23·9	25·8
Means	31·2	32·3	13·3	24·8	26·3	24·0	25·0

* With 80·6 mM sodium citrate.

The effect of the different sugars in the diluent also varied with the use of different sugars in the thawing solution ($P < 0·01$; Table 6). Spermatozoa frozen in both the glucose and raffinose diluents showed highest mean survival when thawed in the solution containing lactose. However, this solution gave the lowest result for spermatozoa frozen in the lactose diluent. The best combination was the use of raffinose in the diluent and lactose in the thawing solution.

High levels of citrate in the diluent proved detrimental to the viability of spermatozoa during post-thawing incubation (level of sodium citrate × duration of incubation, $P < 0·001$; Table 7). The interaction between thawing solutions and

duration of incubation ($P < 0{\cdot}001$; Table 7) appears to be due mainly to the better viability of spermatozoa thawed in the lactose compared with other solutions when assessed at 6 hr.

TABLE 7

EXPERIMENT 4: EFFECTS OF LEVEL OF SODIUM CITRATE IN THE DILUENT AND SUGAR COMPONENT OF THE THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA ASSESSED DURING POST-THAWING INCUBATION

Duration of Incubation (hr)	Sodium Citrate Level in Diluent (mM)			Sugar Component of Thawing Solution (44·4 mM)*			Means
	34	102	170	Glucose	Lactose	Raffinose	
0	35·9	39·0	29·4	33·6	35·8	34·8	34·7
2	32·0	34·7	19·4	28·0	28·3	29·0	28·4
4	28·6	29·6	9·2	21·8	22·5	20·1	21·5
6	28·7	26·4	2·4	17·0	19·3	13·9	16·7
Means	31·2	32·3	13·3	24·8	26·3	24·0	25·0

* With 80·6 mM sodium citrate.

Overall means for each of the four rams were 23·0, 24·1, 26·0, and 27·0 percentage motile spermatozoa ($P < 0{\cdot}01$).

(e) *Experiment 5*

The experiment was of factorial design ($3 \times 2 \times 4 \times 2$) as shown below:

(1) Speed of centrifugation: 0 *v.* 2000 *v.* 3000 r.p.m.
(2) Duration of centrifugation: 5 *v.* 15 min.
(3) Proportion of supernatant removed: 0 *v.* 50 *v.* 75 *v.* 87·5%.
(4) Composition of thawing solution: 88·4 mM sodium citrate *v.* 44·4 mM glucose–80·6 mM sodium citrate.

The pellet volume was 0·03 ml.

None of the main effects concerned with centrifugation, shown in Table 8, were statistically significant. There was, however, a significant interaction between

TABLE 8

EXPERIMENT 5: MEAN PERCENTAGE OF MOTILE SPERMATOZOA FOLLOWING THAWING AND CONCENTRATION BY CENTRIFUGATION

Thawing Solution	Motile Spermatozoa (%)	Speed of Centrifugation (r.p.m.)	Motile Spermatozoa (%)	Duration of Centrifugation (min)	Motile Spermatozoa (%)	Propn. of Supernatant Removed (%)	Motile Spermatozoa (%)	Duration of Incubation (hr)	Motile Spermatozoa (%)
Sodium citrate (88·4 mM)	31·0	0	32·6	5	33·8	0	32·7	0	42·4
		2000	36·0	15	34·4	50	33·3	2	36·0
Glucose (44·4 mM)–sodium citrate (80·6 mM)	37·3	3000	33·8			75	35·8	4	24·5
						87·5	34·4		
P:	< 0·01		n.s.		n.s.		n.s.	< 0·001 (linear) < 0·01 (quadratic)	

speed of centrifugation and duration of incubation on the percentage of motile spermatozoa (0 *v.* 2000 and 3000 r.p.m. × incubation, linear, $P < 0{\cdot}01$). Spermatozoa in treatments that were centrifuged maintained better viability during incubation than those in the control treatment as shown below:

Duration of Incubation (hr)	Speed of Centrifugation (r.p.m.)		
	0	2000	3000
0	43·6	42·9	40·6
2	34·0	36·6	37·5
4	21·1	28·7	23·9

The effect may have been more apparent than real, however, owing to the greater density of the centrifuged samples which could have influenced the assessment of percentage motile spermatozoa.

Spermatozoa thawed in glucose–citrate solution, compared with citrate alone, showed slightly better activity when assessed immediately after thawing and the superiority of the glucose-containing solution became increasingly evident as the incubation progressed (thawing solution × duration of incubation, linear, $P < 0{\cdot}001$) as shown below:

Duration of Incubation (hr)	Thawing Solution	
	Sodium Citrate (88·4 mM)	Glucose (44·4 mM)–Sodium Citrate (80·6 mM)
0	41·5	43·3
2	32·6	39·5
4	19·9	29·4

IV. Discussion

Nagase and Niwa (1964) and Peskovatskii and Habibullin (1968) reported that increasing the volume of pellets from 0·013 to 0·20 ml and from 0·1 to 0·5 ml respectively, had no effect on the revival of bull spermatozoa after pellet freezing. Leipnitz (1966), however, found that 0·1-ml pellets were slightly superior to those 0·2 ml in volume. The experiments reported here clearly show that when freezing ram semen by the pellet method the volume of the pellets can be increased from 0·03 to 0·86 ml without reducing the proportion of spermatozoa that survive the processes of freezing and thawing. In two of three experiments conducted, the post-thawing viability of spermatozoa frozen in large pellets was better than that obtained with small pellets. This result is of practical significance as it enables the preparation of pellets of a size (approximately 0·5 ml in our studies) that will yield sufficient motile spermatozoa for a single insemination.

The cooling curves for pellets of different volume merit some attention (Fig. 2). Rates of cooling between 0 and −60°C (measured by thermocouple* at the centre of the pellet) for pellets of volume 0·032, 0·096, 0·288, and 0·864 ml were approximately 100, 55, 37, and 30 degC/min, respectively. It can be seen, however, that within

* Chromel–Alumel 2ABI (Sodern thermocoax) thermocouple and Philips PR3210U/00 recorder.

any one pellet the rate of cooling is not uniform. It is slower over the temperature range 0 to −5°C and, as little more than half of the semen is in contact with dry ice, cooling is more rapid in the lower portion of the pellet. Even so, the results suggest that using the present methods, approximately threefold variation in the rate of cooling had little effect on the survival of ram spermatozoa after deep-freezing. Nor was there evidence of the occurrence of interactions between rate of cooling and either diluent tonicity or glycerol concentration, both factors which are known to be associated with resistance to cellular damage due to possible physical and chemical effects as ice crystals form and solutes precipitate during freezing (reviewed by Mazur

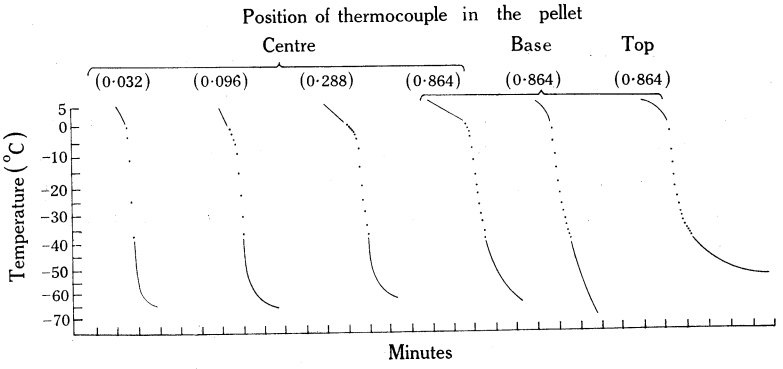

Fig. 2.—Cooling rates during freezing of ram semen by the pellet method in relation to volume of the pellet and position of the thermocouple. Dots from 0 to −40°C are at intervals of 4 sec. Volume of pellet (ml) given in parenthesis.

1966). Most research on the effects of rate of cooling on the survival of spermatozoa after freezing has been conducted with bull semen, although studies in the ram have been reported by Polge (1957), Hill, Godley, and Hurst (1959), Lopatko (1963), Jones (1965), and Entwistle (1968). In general, evidence concerning the optimal rate of cooling is conflicting, a situation which appears to be attributable to the occurrence of interactions between cooling rate and diluent composition, dilution, and equilibration which affect the revival of spermatozoa after freezing.

The procedures that are adopted for thawing spermatozoa frozen by the pellet method can have a marked effect on the proportion of the cells that resume motility. Reports in several species, discussed by Lightfoot and Salamon (1969), have shown that the provision of a solution in which pellets are thawed improves the revival of spermatozoa. This observation is possibly related to the rate of warming, which should be rapid (Salamon 1968). Nagase, Graham, and Niwa (1964) examined a range of solutions for thawing pellet-frozen bull semen but detected no differences as judged by the fertility of the thawed spermatozoa. Subsequently, Essich (1966) reported that the revival of bull spermatozoa was improved by thawing in 3·0% sodium citrate, or milk, compared with 1·5 or 4·5% citrate solutions, and Bock (1968) found better results with saline than a yolk–lactose–glycerol diluent.

Reports on solutions for thawing pellet-frozen ram spermatozoa appear to be limited to that of Salamon (1968) who found that 2·6% sodium citrate gave similar

results to 3·1%, the latter level having been used by Platov (1965, 1966). In the present study the inclusion of a sugar (glucose) in the citrate thawing solution improved both the revival of pellet-frozen ram spermatozoa and subsequent viability during post-thawing incubation. There was further evidence that the use of lactose rather than glucose in the sugar–citrate thawing solution may result in a slightly better survival of spermatozoa frozen in glucose- or raffinose-, but not lactose-containing diluents. A similar type of interaction was reported by Jones and Martin (1965). They found that spermatozoa frozen in a lactose-containing diluent survived post-thawing incubation better after resuspension in fructose-, compared with lactose-containing solutions. Studies on solutions for thawing pellet-frozen semen, however, may be complicated by the fact that the composition of the media can affect both the actual revival of spermatozoa when assessed immediately after thawing, and their viability during subsequent incubation. Thus, in experiment 3, the proportion of spermatozoa that resumed motility when assessed immediately after thawing increased with increasing levels of sodium citrate (from 72·8 to 80·6 mM) in the glucose-containing thawing solution. During subsequent incubation, however, the higher levels of sodium citrate were detrimental to spermatozoal viability. Further studies are needed to find a thawing solution that would maximize both initial revival and survival during incubation.

Dilution rates of 1 : 4 and 1 : 3, prior to freezing and at thawing respectively, reduce the concentration of motile spermatozoa in the thawed sample to approximately $0{\cdot}1 \times 10^9$ cells/ml. If the thawed semen is to be used for insemination, centrifugation is required as a concentration of approximately $1{\cdot}5\text{–}2{\cdot}0 \times 10^9$ motile cells/ml is needed to achieve adequate spermatozoal penetration into the cervix (Lightfoot and Salamon 1970*a*) and fertility (Lightfoot and Salamon 1970*b*; Salamon and Lightfoot 1970). Centrifugation at 3000 r.p.m. for 15 min and removal of up to 87·5% of the supernatant achieves sufficient concentration and does not depress the *in vitro* viability of the spermatozoa.

V. Acknowledgments

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