DEEP FREEZING OF BOAR SEMEN

I. EFFECTS OF DILUENT COMPOSITION, PROTECTIVE AGENTS, AND METHOD OF THAWING ON SURVIVAL OF SPERMATOZOA

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Abstract

A series of factorial experiments was conducted to examine the effects of sugars and polyols (inositol, dulcitol), glycerol, low molecular weight polyols and cell "non-permeating" agents as cryoprotectives, and method of thawing on survival of boar spermatozoa after freezing by the pellet method.

The best recovery rate among the sugars examined (arabinose, xylose, mannose, glucose, lactose, sucrose, raffinose) was obtained with glucose at 315 mM concentration in diluent containing 22·5% egg yolk and 7·5% glycerol. The optimum level for inositol and for dulcitol was 180 and 260 mM respectively.

The low molecular weight polyols (erythritol, xylitol, adonitol) provided some protection to spermatozoa during freezing when included in the diluent at levels from 0·75 to 1·5M, but addition of up to 4·5% glycerol improved the cell survival.

Dextrin, peptone, methylcellulose (Celacol M20), polyvinylpyrrolidone, and ethylenediaminetetraacetic acid in the diluent gave little protection to spermatozoa during the freeze–thaw procedure.

Thawing of pelleted semen in dry test tubes resulted in better cell recovery than thawing in a solution. Thawing at 37°C was superior to thawing at lower temperatures, but there was a little further advantage in increasing thawing temperature to 50 or 60°C.

I. INTRODUCTION

In contrast to the wealth of information concerning frozen storage of bull semen, relatively less information is available on freezing boar semen. The increasing popularity of freezing bull semen by the pellet method (Nagase and Niwa 1964) has stimulated attempts to freeze boar semen by this technique (Rohloff 1967; Kojima *et al.* 1967; Bamba *et al.* 1968*a*, 1968*b*; Grove *et al.* 1968; Dalrymple and Macpherson 1969; Pursel *et al.* 1969; Rohloff and Allmeling 1970; Pursel and Johnson 1971).

Most workers failed in obtaining fertility or obtained very modest and unrepeatable results with deep frozen boar spermatozoa. The best fertility following pellet freezing has been reported by Waide *et al.* (1969), Polge *et al.* (1970), Pursel and Johnson (1971), and Graham *et al.* (1971*b*).

The experiments reported in this paper were conducted prior to the fertility test by Polge *et al.* (1970). The effect was examined of a number of sugars and polyols as the osmotic basis of diluents containing glycerol on the survival of boar spermatozoa

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after freezing by the pellet method. The protective effect of low molecular weight polyols and of several "non-permeating" agents and the conditions of thawing were also examined. An abstract has been published on a part of the results (Salamon *et al.* 1971).

II. Materials and Methods

Semen was collected from Large White, Landrace, and Large Black boars by the manual method using a dummy sow, and only the sperm-rich fraction of the ejaculates was used. The semen samples were diluted 1 : 2 (semen : diluent) at 30°C by one addition of the diluent containing glycerol or low molecular weight polyols (LMWP). The concentrations of egg yolk and of glycerol were 22·5 and 7·5% (v/v) respectively in the diluent, except when glycerol concentration was varied as a factor or was replaced by LMWP. The other diluent components for each experiment are described in Section III.

The diluted semen was cooled to 5°C in 2–2·5 hr, then pelleted (0·09 ml) by dropping the semen from a cooled Pasteur pipette onto dry ice. The semen was kept on dry ice for 2–3 min, then the frozen pellets were transferred into liquid nitrogen and stored for 24–48 hr before thawing.

In the experiments where the temperature of thawing was not included as a factor, the pellets were thawed in test tubes shaken in a water-bath at 37°C. When thawing at temperatures higher than 37°C, the test tubes were held in water-baths at the selected temperatures and then placed in a bath at 37°C immediately after the completion of thawing. In some experiments the pellets were thawed in a solution and the dilution ratio at thawing was 1 : 2 (pellets : thawing solution, v/v). The test tubes into which the pellets were dropped from liquid nitrogen were shaken until thawing was complete.

The percentage of spermatozoa showing progressive movement was assessed under a coverslip on a warm stage (37°C) not later than 5 min following thawing. The assessment was made by one person in all experiments and the identity of samples, all of which were presented in a random order, was not known to the assessor. The test tubes containing the thawed semen were shaken before samples were taken for microscopical examination.

Data were examined by analysis of variance following angular transformation. In the experiments in which semen from individual boars was included and significant first-order interactions involving boars occurred, the interaction mean square was used to test the significance of the relevant main effect. When the ejaculates obtained from boars were pooled and three samples at prefreezing and three samples at thawing (: 3 : 3, expt 6), or two samples at thawing were included (: 2, expt 8), the mean square for within samples at thawing was used as error term.

The results are presented as the retransformed values of the means for the transformed data.

III. Experimental and Results

(*a*) *Experiment 1*

In this experiment ($8\times3\times2$ factorial, using pooled semen from two boars) seven sugars and glycine each at three concentrations were examined (Table 1) and the frozen pellets were thawed at two temperatures (37 and 50°C) without using a thawing solution.

Thawing of pellets in dry test tubes at 37 or 50°C yielded indistinguishable recovery rates (9·5 and 11·5%) and the results have therefore been combined in Table 1. There was an interaction between sugar type and sugar concentration in the diluent ($P<0·001$). While increasing concentration of monosaccharides improved the results, the high levels of di- and trisaccharides had a depressing effect on cell recovery. The monosaccharides gave the best recovery rates at 315 mM concentration.

Lactose and glycine performed best at 210 mM, and sucrose and raffinose at 105 mM concentration.

TABLE 1

EXPERIMENT 1: EFFECTS OF SUGAR TYPE AND SUGAR CONCENTRATION ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Type of sugar in diluent	Identity of sugar	Sugar concn. in diluent (mM)			Means
		105	210	315	
Monosaccharides	Arabinose	1·3	8·0	8·3	5·3
	Xylose	0·2	8·3	15·9	6·3
	Mannose	5·1	9·6	17·9	10·2
	Glucose	6·1	21·3	29·7	17·8
Disaccharides	Lactose	15·0	19·9	5·1	12·6
	Sucrose	17·9	15·0	2·9	10·8
Trisaccharide	Raffinose	16·2	10·9	0·0	6·1
Control	Glycine	10·1	25·0	22·4	18·6
Means		7·5	14·3	10·2	10·5

(*b*) *Experiment 2*

The experiment (4×4×3 factorial) examined the effect of varying concentration of glucose in the diluent and the method of thawing, using ejaculates from three individual boars (Table 2).

TABLE 2

EXPERIMENT 2: EFFECTS OF GLUCOSE CONCENTRATION AND METHOD OF THAWING ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Method of thawing*	Glucose concn. in diluent (mM)				Means
	210	315	420	525	
In dry test tubes	17·9	19·2	6·4	0·57	9·0
In 210 mM glucose solution†	4·3	6·4	5·1	2·3	4·4
In 280 mM glucose solution†	12·8	16·1	11·3	7·9	11·9
In 350 mM glucose solution†	13·2	16·1	13·2	7·9	12·5
Means	11·5	14·0	8·7	3·9	9·1

* Temperature of thawing 37°C.

† Thawing dilution 1 : 2 (pellets : thawing solution, v/v).

The analysis of recovery data revealed an interaction between glucose concentration and method of thawing which was due to a steeper decline of sperm viability with increasing sugar concentration when the pelleted semen was thawed in dry test tubes than in thawing solution [glucose concentration, linear × (in dry test tubes *v.* in thawing solution), $P<0{\cdot}001$]. The best results with all methods of thawing were obtained with 315 mM glucose. Spermatozoa from the three boars differed in freezability ($P<0{\cdot}001$).

(c) *Experiment 3*

The factors included in this experiment (2×4×3×2 factorial, replicated with ejaculates from three boars) were the following:

(1) Polyol type in diluent: inositol *v.* dulcitol.
(2) Polyol concentration in diluent: 100 *v.* 180 *v.* 260 *v.* 340 mM.
(3) Thawing temperature: 37 *v.* 50 *v.* 60°C.
(4) Method of thawing: in dry test tubes *v.* in thawing solution.

The thawing solution consisted of 100 mM inositol and 30 mM sodium citrate.

All factors, except polyol type, had significant effects on the recovery of spermatozoa. The effect of thawing method depended on the thawing temperature ($P<0{\cdot}05$). Table 3 shows that the temperature of thawing had little importance when thawing was performed in dry test tubes, but thawing at 50°C was advantageous when a solution was used for thawing.

TABLE 3

EXPERIMENT 3: INTERACTION BETWEEN THAWING TEMPERATURE AND METHOD OF THAWING ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Method of thawing	Thawing temperature (°C)			Means
	60	50	37	
In dry tubes	22·6	22·2	19·5	21·4
In thawing solution*	11·2	17·8	8·2	12·1
Means	16·5	19·9	13·4	16·5

* Thawing dilution 1 : 2 (pellets : thawing solution, v/v).

There was a second-order interaction ($P<0{\cdot}01$) involving polyol type, polyol concentration, and method of thawing (Table 4). When the pellets were thawed in

TABLE 4

EXPERIMENT 3: RELATIONSHIP BETWEEN TYPE OF POLYOL, POLYOL CONCENTRATION, AND METHOD OF THAWING ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Type of polyol in diluent	Method of thawing*	Polyol concn. in diluent (mM)				Means	
		100	180	260	340		
Inositol	In dry tubes	20·8	27·6	19·8	8·4	18·6	16·0
	In thawing solution†	9·6	14·3	16·7	13·9	13·5	
Dulcitol	In dry tubes	19·7	23·7	30·2	24·3	24·4	17·1
	In thawing solution†	8·4	12·6	10·7	11·8	10·8	
Means		14·1	19·1	18·9	14·2	16·5	

* Temperature of thawing 37°C.
† Thawing dilution 1 : 2 (pellets : thawing solution,/ vv).

dry test tubes the best results with inositol and dulcitol were obtained at 180 and 260 mM concentrations respectively. Further increase in concentration of the two

polyols depressed the recovery rates, but the decline was steeper for inositol than for dulcitol when thawed without a solution. The recovery rates were generally lower when the pellets were thawed in a solution and variation in the concentration of polyol had relatively little effect.

(*d*) *Experiment 4*

This experiment was of $2\times4\times4$ factorial design, replicated with ejaculates from three boars and included the following factors:

(1) Polyol type in diluent: inositol *v.* dulcitol.
(2) Polyol concentration in diluent: 100 *v.* 180 *v.* 260 *v.* 340 mM.
(3) Method of thawing: in dry test tubes
v. in 60 mM inositol–90 mM sodium citrate;
v. in 100 mM inositol–30 mM sodium citrate;
v. in 180 mM inositol.

Table 5 shows that there were significant variations in the recovery rates depending on the type and concentration of polyols and on the method of thawing [(inositol *v.* dulcitol) × polyol concentration × (in dry test tubes *v.* in thawing solution), $P<0\cdot001$].

TABLE 5

EXPERIMENT 4: RELATIONSHIP BETWEEN POLYOL TYPE, POLYOL CONCENTRATION, AND METHOD OF THAWING ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Type of polyol in diluent	Method of thawing*	Polyol concn. in diluent (mM)				Means	
		100	180	260	340		
Inositol	In dry test tubes	15·7	21·6	11·7	0·57	10·4	9·1
	In 60 mM inositol–90 mM citrate†	5·6	8·2	8·2	4·3	6·5	
	In 100 mM inositol–30 mM citrate†	6·7	10·6	12·1	14·9	10·9	
	In 180 mM inositol†	5·1	9·2	14·0	8·9	9·0	
Dulcitol	In dry test tubes	6·4	15·7	19·2	17·0	14·1	8·9
	In 60 mM inositol–90 mM citrate†	9·6	11·3	20·1	10·2	12·6	
	In 100 mM inositol–30 mM citrate†	5·6	4·3	7·3	4·3	5·3	
	In 180 mM inositol†	3·6	4·3	8·2	5·1	5·2	
Means		7·0	10·1	12·3	7·2		9·0
Motile sperm (%) pre-freezing	Inositol diluent	65·0	76·7	58·3	56·7		
	Dulcitol diluent	71·7	63·3	66·7	68·3		

* Temperature of thawing 37°C.
† Thawing dilution 1 : 2 (pellets : thawing solution, v/v).

The best revival rate with inositol was obtained at 180 mM concentration and when thawing solution was not used. Dulcitol performed best at 260 mM level and the recovery rates were much the same when the pellets were thawed in dry test tubes or in 60 mM inositol–90 mM citrate thawing solution.

(*e*) *Experiment 5*

This experiment was of 2×3×4 factorial design, replicated with ejaculates from three boars, and incorporated the following factors:

(1) Basic diluent type: egg yolk–glucose 315 mM in diluent
v. egg yolk–inositol 180 mM in diluent (no glycerol).

(2) Type of LMWP in diluent: adonitol *v.* erythritol *v.* xylitol.

(3) Concentration of LMWP in diluent: 0·25 *v.* 0·50 *v.* 0·75 *v.* 1·0M.

The basic diluents used and the type and concentration of the LMWP had no effects on the motility of spermatozoa during the 2-hr cooling period to 5°C (prefreezing).

The mean revival of spermatozoa after thawing was better in egg yolk–glucose than in egg yolk–inositol diluent ($P<0·01$; Table 6). Erythritol in the diluent provided less protection to spermatozoa during freezing than either xylitol or adonitol, the mean recovery rates for the three polyols being 21·7, 26·0, and 27·4% respectively ($P<0·05$). There was an interaction between type of basic diluent and polyol concentration ($P<0·01$). While recovery of spermatozoa improved with increasing concentration of polyol in the inositol–egg yolk diluent, the results for polyol concentrations in the glucose–egg yolk diluent varied in a curvilinear fashion with best response at 0·50 and 0·75M, and lower response at 0·25 and 1·0M concentrations.

TABLE 6

EXPERIMENT 5: EFFECT OF CONCENTRATION OF LOW MOLECULAR WEIGHT POLYOL (LMWP) IN THE DILUENT ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Type of diluent*	Concn. of LMWP in diluent (M)				Means
	0·25	0·50	0·75	1·00	
Glucose–egg yolk	25·3	29·1	30·7	27·2	28·1
Inositol–egg yolk	14·2	18·8	26·6	30·2	22·1
Means	19·5	23·8	28·6	28·7	25·0

* Containing no glycerol.

(*f*) *Experiment 6*

In this experiment (3× 5 : 3 : 3 factorial design) the low molecular weight polyols (adonitol, erythritol, xylitol), as protective agents during freezing, were examined at wider concentration range (five concentrations) in the egg yolk–inositol (180 mM) diluent. Three samples at prefreezing dilution from pooled ejaculates of two boars and three samples at thawing for each treatment were used.

The mean recovery rates for the three polyols did not differ significantly, but type of polyol and polyol concentration interacted ($P<0·001$). Table 7 shows that while optimum concentration for both adonitol and erythritol was 1·0M, xylitol gave the best and similar results at both 0·5 and 1·0M concentrations. The recovery rates decreased for all polyols when present at concentrations higher than 1·0M.

(g) *Experiment 7*

This experiment (3×6×3 factorial) examined three glycerol concentrations and six xylitol concentrations in the egg yolk–inositol (180 mM) diluent, using ejaculates from three boars.

TABLE 7

EXPERIMENT 6: EFFECTS OF TYPE AND CONCENTRATION OF LOW MOLECULAR WEIGHT POLYOL IN THE DILUENT ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Diluent: egg yolk–inositol 180 mM; no glycerol

Type of polyol	Concn. of polyol in diluent (M)					Means
	0·5	1·0	1·5	2·0	2·5	
Adonitol	16·7	26·0	21·1	14·6	11·3	17·7
Erythritol	16·1	26·5	21·4	16·5	10·0	17·8
Xylitol	22·5	22·5	18·6	13·9	13·9	18·1
Means	18·4	25·0	20·4	15·0	11·7	17·9

There was an interaction between concentration of glycerol and concentration of xylitol ($P<0·001$; Table 8). There were no surviving spermatozoa upon thawing when both glycerol and xylitol were absent or when 7·5% glycerol and 2·5M xylitol were combined. In the absence of glycerol increasing xylitol concentration up to

TABLE 8

EXPERIMENT 7: EFFECT OF CONCENTRATION OF GLYCEROL AND OF XYLITOL ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Diluent: egg yolk–inositol 180 mM

Glycerol concn. in diluent (% v/v)	Xylitol concn. in diluent (M)						Means
	0	0·5	1·0	1·5	2·0	2·5	
0	0·0	14·9	17·4	21·6	20·6	1·8	9·8
3·75 (2·5)*	26·5	23·0	25·0	24·5	23·5	12·5	22·3
7·50 (5·0)*	29·7	28·1	21·6	16·5	6·4	0·0	13·9
Means	13·2	21·7	21·2	20·8	16·0	2·7	15·0

* Concentration in 1 : 2 diluted semen.

1·5M improved the survival rates, but 2·5M proved to be deleterious to the sperm cells. Glycerol gave better protection to spermatozoa than xylitol or the combination of the two agents. The semen of the three boars differed in behaviour to freezing ($P<0·001$).

(h) *Experiment 8*

In this experiment (3×5×3 : 2 factorial) glycerol and xylitol, as cryoprotective agents, were examined at narrower concentration ranges (Table 9), using ejaculates from three individual boars and thawing two samples from each treatment.

The percentages of motile spermatozoa after the 2 hr cooling to 5°C (prefreezing) were similar for the glycerol or xylitol treatments and for their combinations.

The analysis of post-thawing data revealed that xylitol and glycerol concentrations interacted ($P<0{\cdot}001$; Table 9). Only a few spermatozoa survived the freezing when both agents were absent from the diluent. Xylitol at 0·5 and 1·0M concentrations gave equal protection, but the effect was lower than with 1·125–4·5% glycerol concentrations solely. Addition of glycerol to 0·5M and 1·0M xylitol improved the results.

TABLE 9

EXPERIMENT 8: INTERACTION BETWEEN CONCENTRATION OF GLYCEROL AND OF XYLITOL ON THE PERCENTAGE MOTILE SPERMATOZOA FOLLOWING THAWING

Diluent: egg yolk–inositol 180 mM

Xylitol concn. in diluent (M)	Glycerol concn. in diluent (% v/v)					Means
	0	1·125 (0·75)*	2·250 (1·50)*	3·375 (2·25)*	4·500 (3·00)*	
0	5·1	17·9	23·3	23·5	23·5	17·8
0·5	15·1	21·3	21·8	27·3	22·3	21·4
1·0	15·3	22·5	23·5	23·5	26·6	22·2
Means	11·4	20·5	22·9	24·7	24·1	20·5

* Concentration in 1 : 2 diluted semen.

(*i*) *Experiment 9*

The experiment was of 5×3×2×2 factorial design, replicated with ejaculates from three boars, and examined various cell "non-permeating" agents in the glucose (315 mM) diluent in the presence or absence of egg yolk or of glycerol (Table 10).

TABLE 10

EXPERIMENT 9: REVIVAL OF SPERMATOZOA FOLLOWING FREEZING WITH DILUENT CONTAINING "NON-PERMEATING" AGENT AND IN THE ABSENCE OR PRESENCE OF EGG YOLK OR OF GLYCEROL

Basic diluent: glucose 315 mM

Type of agent ($n = 36$)	Motile sperm (%)	Concn. of agent* ($n = 60$)	Motile sperm (%)	Egg yolk ($n = 90$)	Motile sperm (%)	Glycerol ($n = 90$)	Motile sperm (%)	Boar No. ($n = 60$)	Motile sperm (%)
Celacol M20	13·9	1	13·6	Absent	11·1	Absent	8·6	1	13·5
Dextrin	11·8	2	13·1	Present†	16·3	Present‡	19·4	2	15·3
Peptone	13·4	3	14·1					3	12·0
PVP	13·2								
EDTA	15·7								
P	n.s.		n.s.		n.s.		n.s.		<0·05

* See Section III(*i*). † 15% v/v in 1 : 2 diluted semen. ‡ 5% in 1 : 2 diluted semen.

The reason for choosing glucose rather than inositol sulution as a diluent was because in an initial incubation test at 37°C with fresh semen the agents listed below performed better with the former medium.

The agents used and their concentration—indicated under 1, 2, and 3—were the following:

Agent	Concentration in diluent		
	1	2	3
Methylcellulose (Celacol M20)	0·19	0·38	0·76%
Dextrin	1·00	2·00	4·00%
Peptone	1·00	2·00	4·00%
Polyvinylpyrrolidone (PVP)	1·00	2·00	4·00%
Ethylenediaminetetraacetic acid (EDTA)	200	400	800 μM

The five agents gave similar results, and variation in their concentration also had no significant effect on the recovery rates.

The factors egg yolk and glycerol in diluted semen interacted with boars in the analysis of variance ($P<0·05$), so the relevant treatment × boar interaction was used as error term to test the significance of differences between mean recovery rates when either egg yolk or glycerol were present or absent. After using this test both main effects failed to attain statistical significance, due to variations between boars within treatments.

The absence and presence of egg yolk and of glycerol interacted ($P<0·05$) on the percentage motile spermatozoa upon thawing, which is shown below:

Egg yolk	Glycerol	
	Absent	Present
Absent	5·9	17·6
Present	11·8	21·3

Few spermatozoa remained alive when both egg yolk and glycerol were absent and the superiority of protective action of glycerol was clearly evident.

IV. Discussion

Among the sugars examined as diluent components in addition to egg yolk and glycerol for the pellet freezing of boar spermatozoa the best results were obtained with a diluent containing 315 mM–glucose.

Some high molecular weight polyols also proved to be satisfactory diluent components and the optimal concentrations for inositol and dulcitol, which were studied in detail, were 180 and 260 mM respectively. However, none of the high molecular weight polyols (inositol, mannitol, sorbitol, dulcitol) examined in our preliminary tests gave protection to boar spermatozoa during pellet freezing in the absence of glycerol. This is in contrast to the report of Nagase and Tomizuka (1968) who found that sorbitol (0·1–0·5M) in egg yolk–citrate diluent gave reasonable protection to bull spermatozoa. These observations might reflect a difference in permeability of spermatozoa of the bull and the boar to the polyol. It has been found (Drevius 1971, 1972) that bull sperm membrane has a relatively high permeability to sorbitol.

The low molecular weight polyols (adonitol, erythritol, xylitol) do not support motility of non-frozen boar spermatozoa in the absence of other osmotically active agents (Salamon *et al.*, unpublished data). When, in the present study, however, they

were examined as alternative protective agents to glycerol, it was found that adonitol, erythritol, and xylitol provided considerable protection to boar spermatozoa during pellet freezing, thus extending the observations of Nagase (1968) and of Nagase and Tomizuka (1968) with bull spermatozoa. These polyols were most effective in protecting the sperm cells when present in the diluent at 0·75–1·5M concentration. When the absence and presence of xylitol and of glycerol or combinations of these two compounds in the diluent were examined, however, glycerol was clearly superior as a protective agent and the presence of the low molecular weight polyol seemed to have no additional effect. Nevertheless, in the light of findings that glycerol reduces the fertilizing capacity of boar spermatozoa (Polge 1956; King and Macpherson 1966; Leidl 1968; Neville *et al.* 1970; Graham *et al.* 1971*a*) fertility trials with boar semen frozen in diluents containing low molecular weight polyols as cryoprotective agents seem warranted.

The cell "non-permeating" agents (Celacol M20, dextrin, peptone, PVP) at the concentrations used in the present study gave little protection to the sperm cells during the freeze–thaw procedure (as assessed on post-thawing motility). The EDTA, reported to be beneficial under liquid storage conditions of boar semen (Plisko 1965; Sadovnikova 1966; Doroskov and Kistymova 1966) was also of negligible value as a protective agent. Although an effect on cell motility after freezing and thawing could not be demonstrated, some of these compounds may provide additional protection to the cell membrane when used in conjunction with other cryoprotective agents. The effect of these compounds on the retention of fertilizing capacity of frozen–thawed boar spermatozoa remains therefore to be investigated.

In the present study only the results for revival of spermatozoa upon thawing were presented, although in most experiments survival of spermatozoa during incubation at 37°C following thawing was also examined. Due to very poor or, in many instances, no cell survival after incubation for 1 hr only data for recovery upon thawing were analysed. Other workers (King and Macpherson 1967; Dalrymple and Macpherson 1969) also reported poor post-thawing survival and have considered this as one of the possible reasons for the lack of fertility of frozen boar spermatozoa. Recently, however, Polge *et al.* (1970) found that a proportion of pellet frozen and thawed boar spermatozoa retained their fertilizing capacity when deposited into the sow's oviduct as early as 20 hr before ovulation, and maintained their motility in the oviduct for 24 hr.

One of the possible causes of low survival *in vitro* could be a thermosensitivity of sperm cells weakened during freezing to prolonged exposure to 37°C or higher temperature. On the other hand, the frozen pellets should be thawed at 37°C or higher temperature in order to obtain maximum cell recovery. In our additional experiments (not presented here) thawing at temperature ranges below 37°C resulted in very poor recovery rates. It is peculiar that, although the pellet frozen boar spermatozoa require a relatively fast thawing, the use of a thawing solution—which could increase the velocity of thawing—was not advantageous. This is in contrast to findings in other species (reviewed by Lightfoot and Salamon 1969*a*, 1969*b*) that the use of a solution for thawing the pellet frozen semen resulted in better sperm recovery than thawing without solution. At the present time no explanation can be offered for the different behaviour of boar spermatozoa.

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