EFFECT OF COMPOSITION OF TRIS-BASED DILUENT AND OF THAWING SOLUTION ON SURVIVAL OF RAM SPERMATOZOA FROZEN BY THE PELLET METHOD

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Abstract

Six factorial experiments were conducted to examine the effects of concentration of tris(hydroxymethyl)aminomethane (Tris) and type and concentration of sugar in the freezing diluent, and of composition of thawing solution on the survival of ram spermatozoa following the freeze–thaw procedure.

Spermatozoa tolerated a relatively wide range in concentration of Tris, but the cell survival varied depending on the type of sugar included in the Tris diluent and on the composition of the thawing solution. Inclusion of sugar in the freezing diluent was beneficial with glucose a more suitable component than fructose, lactose, or raffinose. When the latter sugars were included in the freezing diluent, the choice of thawing solution was more critical than for the Tris–glucose diluent.

The best results were obtained using Tris (300 mM)–glucose (27·75 mM)–yolk (15% v/v)–glycerol (5% v/v) freezing diluent (dilution 1 : 4, semen : diluent) and Tris (300 mM)–fructose (55·5 mM) thawing solution (dilution ratio 1 : 3, pellets : thawing solution, v/v).

I. INTRODUCTION

Diluents containing tris(hydroxymethyl)aminomethane (Tris) as a main component have been examined for frozen storage of bull (Davis, Bratton, and Foote 1963; Yassen and Foote 1967; Hahn, Cassou, and Eibl 1969; Foote 1970*a*, 1970*b*) and boar semen (Rohloff 1967; Grove, Bollwahn, and Mahler 1968). The use of Tris-based diluent for pellet-freezing ram spermatozoa appears to be reported only by Samouilidis (1970).

The experiments reported here were conducted to examine the effects of Tris concentration and type and concentration of sugar in the freezing diluent and of composition of the thawing solution on the survival of ram spermatozoa following the freeze–thaw procedure.

II. MATERIALS AND METHODS

Semen was collected from mature Merino rams by artificial vagina. In the experiments in which rams were included as a factor, either one (expt. 6) or two (expts. 1, 2, 5) ejaculates were collected from each ram. In the latter case, two ejaculates from individual rams were pooled before dilution. In experiments 3 and 4, ejaculates collected from seven rams were pooled prior to use. Aliquots of semen were diluted (1 : 4) at 30°C by a single addition of the glycerol-containing diluent.

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The sugar component of Tris-based diluent was 55·50 mM fructose (Steinbach and Foote 1967) in experiments 1 and 2, 27·75 mM glucose in experiment 6, 27·75 mM fructose or glucose in experiment 3, and was varied in experiments 4 and 5. The pH of Tris-based diluent in each experiment was adjusted to 7·0 with an appropriate amount of citric acid. Each diluent contained 15% (v/v) egg yolk. The glycerol concentration in the diluted semen prior to freezing was 4% (v/v), except in experiment 2 where different glycerol concentrations were examined.

The diluted semen was cooled to 5°C in 2 hr and held at this temperature for an additional 1 hr before pelleting (0·06 ml) on dry ice (Nagase and Niwa 1963, 1964). The semen was kept on dry ice for 3 min, after which the frozen pellets were transferred into liquid nitrogen and stored for 24–72 hr before thawing for examination.

The pellets were thawed in test tubes containing thawing solution (without egg yolk) and held in a water-bath at 37°C. Dilution ratio at thawing was 1 : 3 (pellets : thawing solution, v/v) in all experiments.

The percentage of motile spermatozoa was assessed under coverslip on a warm stage (37°C) immediately after thawing, and at intervals of 2 hr during subsequent incubation for 6 hr at 37°C. Freezing point depression (FPD, degC) of diluents (non-glycerolated) used in experiments 1, 2, and 4 was determined by a Fiske cryoscope. FPD higher than 1·0 could not be measured accurately.

Data for each experiment, following angular transformation, were examined by analyses of variance for a split-plot experiment, post-thawing incubation being the sub-plot. Where significant first-order interaction was revealed between rams and other factors, the interaction mean square was used to test the relevant main effect.

TABLE 1

EXPERIMENT 1: EFFECT OF CONCENTRATION OF TRIS IN THE DILUENT AND IN THE THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Tris concn. in thawing solution* (mM)	Tris concn. in diluent* (mM)				Time of incubation (hr)				Means
	150 (0·47)†	250 (0·66)†	350 (0·85)†	450 (1·03)†	0	2	4	6	
150	20·1	27·6	23·0	15·7	26·9	22·4	20·3	16·5	21·4
250	31·5	42·5	41·2	34·2	45·7	38·3	34·9	30·6	37·3
350	29·9	35·2	38·6	31·6	41·8	37·4	32·4	24·2	33·8
450	22·1	27·4	24·2	19·9	36·4	29·7	20·6	10·0	23·3
Means	25·7	33·0	31·5	24·9	37·6	31·7	26·8	19·7	

* Combined with 55·5 mM fructose.

† Freezing point depression of non-glycerolated portion (degC).

III. EXPERIMENTAL AND RESULTS

(a) *Experiment 1*

The experiment was of 4×4×4 factorial design and examined the following factors:

(1) Concentration of Tris in freezing diluent: 150 *v.* 250 *v.* 350 *v.* 450 mM.*

(2) Concentration of Tris in thawing solution: 150 *v.* 250 *v.* 350 *v.* 450 mM.*

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

* Each concentration combined with 55·5 mM fructose.

There were significant interactions between concentrations of Tris in the diluent and thawing solution ($P < 0{\cdot}01$), and between Tris concentration in the thawing solution and duration of incubation ($P < 0{\cdot}001$; Table 1). Low (150 mM) and high (450 mM) concentrations of Tris in either diluent or thawing solution depressed the survival of spermatozoa. Combination of 250 and 350 mM concentrations of Tris in both diluent and thawing solution improved the results, the best being achieved when the thawing medium contained 250 mM Tris. This solution gave the best recovery on thawing and the best survival of spermatozoa during post-thawing incubation.

The analysis of variance revealed a second-order interaction involving concentration of Tris in both diluent and thawing solution and post-thawing incubation time ($P < 0{\cdot}05$). Nevertheless, the best survival rates were observed when the semen was frozen with 250 and 350 mM Tris diluent, and subsequently thawed in 250 mM Tris solution.

There was a significant difference between rams ($P < 0{\cdot}001$).

TABLE 2

EXPERIMENT 2: RELATIONSHIPS BETWEEN GLYCEROL CONCENTRATION, TRIS CONCENTRATION IN DILUENT, AND TYPE OF THAWING SOLUTION ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Glycerol concn. in diluted semen (%)	Tris concn. in diluent* (mM)			Thawing solution			Means
	250 (0·66)†	400 (0·94)†	550 (>1·0)†	Inositol–citrate	Fructose	Tris–fructose	
0	9·4	15·3	7·2	14·5	12·6	5·2	10·4
2	31·0	36·1	21·4	32·9	27·9	27·1	29·3
4	37·7	39·5	25·1	39·0	35·7	27·3	33·9
Means	24·7	29·6	17·0	28·1	24·7	18·4	

* Combined with 55·5 mM fructose.

† Freezing point depression of non-glycerolated portion (degC).

(*b*) *Experiment 2*

The factors included in this experiment ($3 \times 3 \times 3 \times 3$ factorial) were the following:

(1) Concentration of Tris in freezing diluent: 250 *v.* 400 *v.* 550 mM.*

(2) Glycerol concentration in diluted semen: 0 *v.* 2 *v.* 4% (v/v).

(3) Thawing solution: inositol (210 mM)–citrate (40 mM) *v.* fructose (388·5 mM) *v.* Tris (250 mM)–fructose (55·5 mM).

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

* Each concentration combined with 55·5 mM fructose.

All factors examined had significant effects ($P < 0{\cdot}001$) on the mean percentage of motile spermatozoa assessed during post-thawing incubation. Glycerol concentration interacted with Tris concentration in the diluent and with type of thawing solution (Table 2). In the former case, survival of spermatozoa improved with increasing concentrations of glycerol in the diluent for all Tris concentrations, but the effect was less with 550 mM than with 250 and 400 mM Tris diluents (glycerol concentration $\times$ Tris concentration; $P < 0{\cdot}001$).

Few spermatozoa survived when the semen frozen in the absence of glycerol was subsequently thawed in Tris–fructose solution, which seemed to have no "repairing" action on the sperm cells protected to a similar degree by 2 and 4% glycerol concentrations during freezing. However, when the semen was frozen without glycerol and thawed in inositol–citrate or fructose solution, relatively more spermatozoa maintained their viability, and the beneficial effect of both thawing solutions seemed to be additive to the protective action of increasing glycerol concentration (glycerol concentration $\times$ thawing solution; $P < 0{\cdot}001$).

There was a further significant interaction ($P < 0{\cdot}001$) between Tris concentration and type of thawing solution (Fig. 1). Spermatozoa frozen with 250 mM Tris

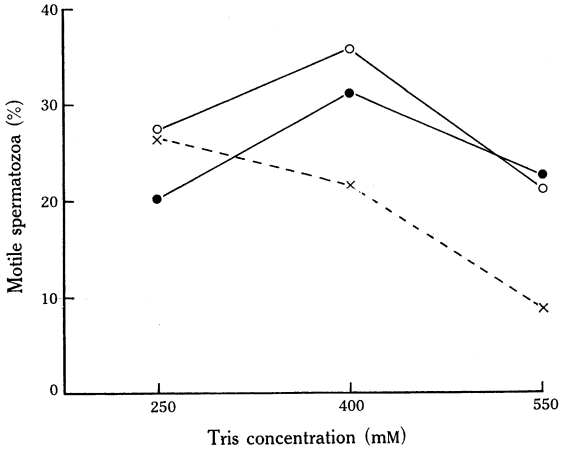

Fig. 1.—Experiment 2: Effect of concentration of Tris in diluent and of type of thawing solution on the mean percentage of motile spermatozoa during post-thawing incubation.

○ Inositol–citrate.

● Fructose.

× Tris–fructose.

diluent survived better when thawed in inositol–citrate and Tris–fructose than in fructose solution. Increase in tonicity of the diluent depressed the survival of cells thawed in Tris–fructose solution, while viability improved for 400 mM and decreased for 550 mM Tris extender when inositol–citrate and fructose thawing media were used.

The post-thawing viability of spermatozoa was influenced by concentration of Tris in the diluent ($P < 0{\cdot}01$) and type of thawing solution ($P < 0{\cdot}001$) and these factors were also involved in a second-order interaction (Tris concentration $\times$ thawing solution $\times$ time of incubation; $P < 0{\cdot}001$), shown in Table 3. Decline in viability of spermatozoa during post-thawing incubation showed a similar pattern for all three Tris diluents (250, 400, 550 mM) when the semen was thawed and incubated in

inositol–citrate or Tris–fructose solution. However, when the frozen pellets were thawed in fructose solution the percentage of motile spermatozoa decreased more steeply during incubation for all freezing diluents, and particularly for 250 mM Tris.

TABLE 3

EXPERIMENT 2: EFFECT OF CONCENTRATION OF TRIS IN DILUENT AND OF TYPE OF THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Thawing solution	Time of incubation (hr)	Tris concn. in diluent* (mM)			Means
		250	400	550	
Inositol–citrate	0	35·7	45·9	27·2	36·1
	2	31·3	37·3	23·2	30·4
	4	24·2	32·4	18·4	24·8
	6	20·0	29·0	16·8	21·7
Means		27·6	36·0	21·3	28·1
Tris–fructose	0	32·4	25·8	15·0	24·0
	2	29·3	23·7	11·2	20·8
	4	25·2	20·1	5·9	16·1
	6	20·0	17·9	4·7	13·3
Means		26·6	21·8	8·8	18·4
Fructose	0	38·5	44·2	32·3	38·3
	2	21·1	32·2	22·7	25·2
	4	14·3	26·4	19·2	19·7
	6	11·0	24·3	17·3	17·2
Means		20·3	31·5	22·7	24·7
Overall means		24·7	29·6	17·0	

* Combined with 55·5 mM fructose.

(c) *Experiment 3*

Tris extender containing no sugar or combined with fructose or glucose was included in this $3 \times 3 \times 4 \times 3$ factorial experiment with the following factors:

(1) Concentration of Tris in freezing diluent: 200 *v.* 300 *v.* 400 mM.

(2) Sugar in diluent: nil *v.* fructose (27·75 mM) *v.* glucose (27·75 mM).

(3) Thawing solution: Tris (250 mM)–fructose (55·5 mM) *v.* Tris (250 mM)–glucose (55·5 mM) *v.* inositol (210 mM)–citrate (40 mM) *v.* fructose (388·5 mM).

(4) Replicate: three replicates from pooled semen of seven rams.

All factors, except replicate, had significant effects ($P < 0{\cdot}001$) on the mean percentage of motile spermatozoa following thawing. Inclusion of sugar in the diluent was beneficial and the best mean results were obtained with Tris–glucose extender. There was a second-order interaction ($P < 0{\cdot}05$) involving concentration of Tris and sugar in the freezing diluent and the type of thawing solution (Table 4). Tris–fructose

TABLE 4

EXPERIMENT 3: EFFECTS OF TRIS CONCENTRATION AND SUGAR IN DILUENT AND OF THAWING SOLUTION ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Sugar in diluent	Thawing solution	Tris concn. in diluent (mM)			Means
		200	300	400	
Nil	Tris–fructose	30·1	34·1	25·0	29·7
	Tris–glucose	28·1	32·9	27·3	29·4
	Inositol–citrate	21·1	29·3	33·3	27·7
	Fructose	7·1	27·0	23·6	18·2
Means		20·6	30·8	27·2	26·1
Fructose	Tris–fructose	32·9	35·4	23·5	30·5
	Tris–glucose	35·4	34·1	27·7	32·3
	Inositol–citrate	32·5	35·4	32·1	33·3
	Fructose	11·1	24·9	28·1	20·8
Means		27·2	32·3	27·8	29·1
Glucose	Tris–fructose	35·8	33·7	28·1	32·5
	Tris–glucose	37·1	33·3	30·5	33·6
	Inositol–citrate	31·3	38·3	39·2	36·2
	Fructose	24·2	32·9	35·8	30·9
Means		32·0	34·5	33·3	33·3
Overall means		26·5	32·5	29·4	

and Tris–glucose solutions were more suitable thawing media for spermatozoa frozen with 200 or 300 mM Tris than with 400 mM Tris, without sugar or combined with either fructose or glucose. The results for inositol–citrate thawing solution improved with increasing Tris concentrations when sugar was absent or glucose was present in the Tris diluent. Fructose solution was a particularly poor thawing medium for spermatozoa frozen with 200 mM Tris without sugar or in combination with fructose.

Viability of spermatozoa during post-thawing incubation was influenced by each concentration of Tris and type of sugar in diluent ($P < 0{\cdot}05$) and by type of

thawing solution ($P < 0{\cdot}001$). These factors were also involved in second-order interactions ($P < 0{\cdot}01$, $P < 0{\cdot}001$).

(*d*) *Experiment 4*

The experiment was of $3 \times 4 \times 3 \times 3$ factorial design and examined the following factors:

(1) Concentration of Tris in freezing diluent: 150 *v.* 300 *v.* 450 mM.

(2) Sugar in diluent: glucose *v.* fructose *v.* lactose *v.* raffinose.

(3) Concentration of sugar in diluent: 27·75 *v.* 55·50 *v.* 83·25 mM.

(4) Thawing solution: inositol (210 mM)–citrate (40 mM) *v.* Tris (250 mM)–fructose (55·5 mM) *v.* fructose (388·5 mM).

The mean percentages of motile spermatozoa for the three sugar concentrations in the diluent were similar (27·0, 26·9, 27·8), but there was an interaction between concentrations of sugar and of Tris ($P < 0{\cdot}001$; Fig. 2). Increasing sugar concentration in 150 mM Tris was beneficial, but it slightly depressed viability of spermatozoa in 300 and 450 mM Tris diluents. There were also significant interactions between Tris concentration and type of sugar in the diluent ($P < 0{\cdot}001$) and between type of sugar in the diluent and the composition of thawing solution ($P < 0{\cdot}001$), and these factors were involved in a second-order interaction ($P < 0{\cdot}05$). Table 5 shows that the optimum concentration of Tris in the diluent was 300 mM which, combined with any of the sugars examined (glucose, fructose, lactose, or raffinose) and with all three thawing solutions, gave better results than 150 and 450 mM Tris concentrations. Within the 300 mM Tris combinations, best results were obtained with glucose as the sugar in the diluent, and with fructose thawing solution.

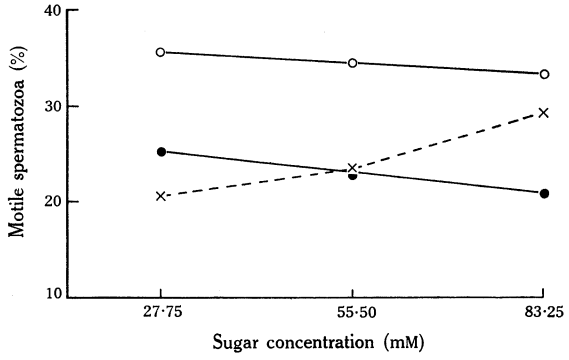

Fig. 2.—Experiment 4: Effect of concentration of Tris and sugar in diluent on the mean percentage of motile spermatozoa during post-thawing incubation.

● 450 mM Tris.

○ 300 mM Tris.

× 150 mM Tris.

Each factor—concentration of Tris and type of sugar in the diluent and composition of the thawing solution—had an influence ($P < 0{\cdot}001$) on the viability of spermatozoa during post-thawing incubation, and there were two second-order interactions (Table 6).

First, viability of spermatozoa in all three thaw–incubation solutions was maintained best when 300 mM Tris diluent was used for freezing. When spermatozoa were frozen with 150 mM Tris extender, viability during post-thawing incubation was better in inositol–citrate and Tris–fructose than in fructose solution. Both inositol–citrate and fructose solutions, however, were more suitable than Tris–fructose as thaw–incubation media when a diluent of high tonicity (450 mM Tris) was used for freezing.

TABLE 5

EXPERIMENT 4: EFFECTS OF TRIS CONCENTRATION AND SUGAR TYPE IN DILUENT AND OF THAWING SOLUTION ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Sugar in diluent	Thawing solution	Tris concn. in diluent (mM)			Means
		150 (0·41–0·56)*	300 (0·70–0·87)*	450 (>1·0)*	
Glucose	Inositol–citrate	26·9	37·1	32·9	32·2
	Tris–fructose	28·3	37·9	23·4	29·7
	Fructose	24·7	39·2	37·5	33·6
Means		26·6	38·0	31·1	31·8
Fructose	Inositol–citrate	26·9	35·0	26·1	29·3
	Tris–fructose	28·1	33·3	16·8	25·7
	Fructose	7·6	31·7	26·8	20·8
Means		19·8	33·3	23·0	25·2
Lactose	Inositol–citrate	31·3	35·8	22·9	29·8
	Tris–fructose	30·9	30·1	15·4	25·0
	Fructose	17·8	36·6	22·8	25·3
Means		26·4	34·1	20·2	26·7
Raffinose	Inositol–citrate	27·3	30·9	21·4	26·4
	Tris–fructose	30·9	32·1	13·6	25·0
	Fructose	18·1	35·0	22·2	24·7
Means		25·2	32·6	18·9	25·4
Overall means		24·4	34·5	23·2	

* Range in freezing point depression when the Tris concentration was combined with 27·75, 55·50, and 83·25 mM sugars.

Secondly, the pattern of decline in viability of spermatozoa during post-thawing incubation was similar for all thawing media when Tris diluent containing glucose was used for freezing. When fructose, lactose, or raffinose was present in the freezing

diluent, decrease in survival of spermatozoa during incubation was steeper in fructose than in inositol–citrate and Tris–fructose thaw–incubation solutions, particularly following freezing with the diluent containing fructose.

TABLE 6

EXPERIMENT 4: RELATIONSHIPS BETWEEN COMPOSITION OF THAWING SOLUTION, CONCENTRATION OF TRIS, AND TYPE OF SUGAR IN THE DILUENT ON THE PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Interactions: Tris concentration in diluent × thawing solution × time of incubation ($P < 0{\cdot}001$); sugar in diluent × thawing solution × time of incubation ($P < 0{\cdot}001$)

Thawing solution	Time of incubation (hr)	Tris concn. in diluent (mM)			Sugar in diluent				Means
		150	300	450	Glucose	Fructose	Lactose	Raffinose	
Inositol–citrate	0	35·8	42·6	35·8	37·9	38·5	38·5	37·3	38·0
	2	28·5	34·5	25·2	34·0	28·6	29·1	25·8	29·4
	4	25·4	33·3	22·8	31·8	26·5	27·4	22·7	27·0
	6	23·1	28·5	19·9	25·5	24·0	24·8	20·8	23·8
Means		28·1	34·6	25·7	32·2	29·3	29·8	26·4	29·4
Tris–fructose	0	35·0	37·9	24·5	35·7	31·8	30·6	31·3	32·3
	2	32·1	35·4	20·1	32·3	27·9	28·4	27·2	29·0
	4	27·3	31·3	16·0	28·6	25·3	21·6	22·9	24·5
	6	24·1	28·9	9·5	22·7	18·4	20·1	19·1	20·1
Means		29·5	33·3	17·1	29·7	25·7	25·0	25·0	26·3
Fructose	0	31·7	43·5	38·7	39·6	37·9	36·8	37·3	37·9
	2	16·0	35·4	26·4	34·5	19·5	24·8	23·8	25·5
	4	13·5	33·3	24·0	32·3	17·0	22·4	21·6	23·1
	6	8·1	30·5	20·3	28·2	11·7	18·4	17·6	18·6
Means		16·5	35·6	27·1	33·6	20·8	25·3	24·7	26·0
Overall means		24·4	34·5	23·2	31·8	25·2	26·7	25·4	

(*e*) *Experiment 5*

The 300 mM concentration of Tris in the freezing diluent, found to be optimum in experiments 3 and 4, was combined with glucose, fructose, or lactose in a $3 \times 8 \times 3$ factorial experiment with the following factors:

(1) Sugar in freezing diluent: glucose *v.* fructose *v.* lactose (each at 27·75 mM).

(2) Thawing solution: eight solutions shown in Table 7.

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

TABLE 7

EXPERIMENT 5: EFFECT OF COMPOSITION OF FREEZING DILUENT AND OF THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Time of incubation (hr)	Freezing diluent: Tris–glucose	Tris–fructose	Tris–lactose	Means
0	44·5	44·3	44·1	44·3
2	40·5	40·7	38·1	39·7
4	38·5	37·3	35·4	37·1
6	37·1	34·5	32·2	34·6
Means	40·1	39·2	37·4	

	Thawing solutions: Glucose (55·5 mM) Tris concn. (mM) 250	300	350	Fructose (55·5 mM) Tris concn. (mM) 250	300	350	Inositol (210 mM) –citrate (40 mM)	Fructose (388·5 mM)	
0	47·1	44·8	43·0	44·8	44·2	44·8	44·2	41·9	44·3
2	41·9	39·0	40·7	41·3	41·9	39·6	39·6	34·0	39·7
4	39·0	38·5	36·8	38·5	40·7	37·9	36·8	28·6	37·1
6	36·2	36·2	35·1	36·2	38·5	34·5	35·1	25·2	34·6
Means	41·0	39·6	38·9	40·2	41·3	39·2	38·9	32·3	
	39·86			40·24					

TABLE 8

EXPERIMENT 6: EFFECT OF FREEZING DILUENT AND OF THAWING SOLUTION ON THE PERCENTAGE OF SURVIVING SPERMATOZOA

Freezing diluent	Time of incubation (hr)	Thawing solution: Inositol–citrate	Tris–fructose	Fructose	Means
Raffinose–citrate	0	44·0	47·0	44·8	45·3
	2	37·4	41·1	37·4	38·6
	4	33·1	40·3	30·3	34·5
	6	30·3	39·6	23·5	31·0
Means		36·1	42·0	33·8	37·3
Tris–glucose	0	50·0	49·2	44·0	47·7
	2	45·5	44·0	41·1	43·5
	4	40·3	43·3	38·1	40·6
	6	38·1	41·1	34·5	37·9
Means		43·5	44·4	39·4	42·4
Overall means		39·8	43·2	36·6	

Recovery rates on thawing were similar for the three freezing diluents, but the decline in viability of spermatozoa during subsequent incubation was steeper for Tris–fructose ($P < 0{\cdot}05$) and Tris–lactose ($P < 0{\cdot}01$) than for Tris–glucose.

The three Tris concentrations (250, 300, 350 mM) combined with either glucose or fructose in the thawing solution yielded similar mean results, which were indistinguishable from inositol–citrate solution. Fructose thawing solution showed the poorest mean performance (fructose *v.* rest, $P < 0{\cdot}001$) and proved to be less suitable milieu for maintenance of cell viability during post-thawing incubation (fructose *v.* rest × time of incubation, linear, $P < 0{\cdot}001$). The semen of the three rams differed in resistance to the freeze–thaw procedure ($P < 0{\cdot}001$).

(*f*) *Experiment 6*

This experiment was of $2 \times 3 \times 7$ factorial design and examined the following factors:

(1) Type of freezing diluent: Tris (300 mM)–glucose (27·75 mM) *v.* raffinose (166·5 mM)–citrate (68 mM).

(2) Thawing solution: inositol (210 mM)–citrate (40 mM) *v.* Tris (300 mM)–fructose (55·5 mM) *v.* fructose (388·5 mM).

(3) Rams: ejaculates from seven individual rams.

The results are summarized in Table 8. The mean percentage of motile spermatozoa following the freeze–thaw procedure was better for Tris–glucose than for raffinose–citrate diluent ($P < 0{\cdot}001$). Tris–fructose thaw–incubation solution was more effective than inositol–citrate ($P < 0{\cdot}01$) and fructose ($P < 0{\cdot}001$) solutions. There was a second-order interaction involving freezing diluent, thawing solution, and time of incubation ($P < 0{\cdot}01$), due to a steeper decline in viability of spermatozoa during incubation when the semen frozen in raffinose–citrate was subsequently thawed in fructose solution. The semen of individual rams differed in freezability ($P < 0{\cdot}05$), but there were no interactions with treatments.

IV. Discussion

The main points emerging from the present study are:

(1) ram spermatozoa can tolerate a relatively wide range in Tris concentration in the freezing diluent;

(2) the type of sugar included has an influence on the value of Tris–sugar as a diluent; and

(3) the performance of Tris–sugar freezing diluent is influenced by the composition of the solution used for thawing the frozen pellets.

Steinbach and Foote (1967) examined Tris concentrations ranging from 150 to 350 mM for freezing bull spermatozoa by the conventional method and found that 200 mM was the optimum concentration in the Tris–fructose–yolk diluent adjusted to pH 6·5. Tris at 350 mM concentration in the extender was unsuitable for freezing bull spermatozoa. In the present experiments the Tris-based diluents used for freezing ram spermatozoa were adjusted to pH 7·0, because in our preliminary investigations Tris media with either lower or higher pH decreased post-thawing cell survival.

Ram spermatozoa tolerated a relatively wide range in tonicity of Tris freezing medium but the most acceptable rates of cell survival following the freeze–thaw procedure were observed with Tris concentrations from 250 to 400 mM in the diluent.

Inclusion of sugar in the Tris diluent was beneficial and, of the sugars examined, glucose proved to be more suitable component than fructose, lactose, or raffinose. In the work with semen of the bull (Steinbach and Foote 1967) and ram (Samouilidis 1970) only fructose at one concentration (55·5 mM) was included in the Tris extender. By examining the effect of sugar concentrations here (Fig. 2) it became evident that the amount of sugar required in hypertonic Tris extenders was relatively small (27·75 mM) and higher sugar concentration had a depressing effect on the survival of spermatozoa following the freeze–thaw process. In hypotonic Tris media (150 mM, FPD = 0·41–0·56; FPD of ram semen = 0·64) increasing sugar concentrations improved the cell recovery and concentrations higher than 83·25 mM—the highest concentration examined—could have given further improvement.

Examination of freezing diluents by the inclusion of a thawing solution was made in the light of findings that pellet frozen spermatozoa of the ram (Platov 1965; Salamon 1968), bull (Essich 1966; Bock 1968), jackass and stallion (Krause and Grove 1967) show poor revival when thawed without a thawing solution, and that increasing prefreezing dilution of semen could compensate only partly for the benefit gained by using a thawing medium (Lightfoot and Salamon 1969*a*). Concomitant examination of both freezing and thawing media are supported also by the observation that a relationship exists between their composition (Lightfoot and Salamon 1969*b*). Further evidence concerning such a relationship is presented in the present study, indicating that variation in the constituents of the freezing diluent could require a change in the composition of the thawing solution in order to obtain maximum cell recovery. An appropriate thawing solution may offer some kind of "repairing" action, even to spermatozoa not lethally injured during freezing in the absence of glycerol, and such effect could be additive to the protection given by a glycerol-containing diluent (Table 2). Although there are difficulties in measuring the protective (and other) actions of the freezing diluent and of the thawing solution, the fact that the effects of the two media are linked in the freeze–thaw events indicates that one should also take into account the composition of the thawing solution when assessing the value of diluent(s) used for pellet freezing.

Concerning the sugar component in Tris-based diluents of different tonicity, it should be mentioned that when glucose was present in the diluting medium the choice of thawing solution was less critical than when fructose, lactose, or raffinose was combined with Tris (expt. 4). When, however, the optimum Tris concentration (300 mM) was combined with glucose, fructose, or lactose in the freezing diluent, spermatozoa performed equally well in thawing solutions consisting of inositol–citrate or of 250 to 300 mM Tris combined with either glucose or fructose (expt. 5). The relatively high recovery of spermatozoa on thawing, but the steep decline in cell viability during post-thawing incubation when fructose thawing solution was used, confirms earlier observations (Lightfoot and Salamon 1969*b*; Salamon and Brandon 1971) that a solution which acts beneficially at thawing is not necessarily the optimal milieu for maintaining the viability of cells.

In the final experiment, the Tris–glucose freezing diluent containing its components at the optimal concentration was compared with the raffinose–citrate diluent elaborated earlier (Lightfoot and Salamon 1969*a*) and used in a fertility test (Salamon 1971). The Tris–glucose diluent was the more effective for pellet freezing, and the Tris (300 mM)–fructose (55·5 mM) solution was a better thawing medium than the inositol–citrate and fructose solutions developed previously (Salamon and Brandon 1971). The 300 mM Tris–27·75 mM glucose–15% (v/v) egg yolk shown to be an efficient extender for pellet freezing ram semen has a degree of hypertonicity (FPD 0·72) very similar to that of the raffinose–citrate–yolk diluent (FPD 0·75).

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VI. References

Bock, K. (1968).—Zur Anwendung von Tiefkühlsperma in Pillenform (Pellets). *Dt. tierärztl. Wschr.* **75**, 114.

Davis, I. S., Bratton, R. W., and Foote, R. H. (1963).—Livability of bovine spermatozoa at 5, −25, and −85°C in Tris-buffered and citrate-buffered yolk–glycerol extenders. *J. Dairy Sci.* **46**, 333.

Essich, G. (1966).—Zur Tiefkühlkonservierung von Bullensperma in Pillenform und in kleine Kunststoffröhrchen. Vet.-med. Dissertation, Tierärztl. Fak. Ludwig-Maximilians-Univ., München. [*Anim. Breed Abstr.* **36**, No. 341 (1968).]

Foote, R. H. (1970*a*).—Fertility of bull semen at high extension rates in Tris-buffered extenders. *J. Dairy Sci.* **53**, 1475.

Foote, R. H. (1970*b*).—Influence of extender, extension rate, and glycerolating technique on fertility of frozen bull semen. *J. Dairy Sci.* **53**, 1478.

Grove, D., Bollwahn, W., and Mahler, R. (1968).—Versuche zur Gewinnung und Tiefgefrierkonservierung von Nebenhodenschwanzsperma beim Eber. *Dt. tierärztl. Wschr.* **75**, 35.

Hahn, R., Cassou, B., and Eibl, K. (1969).—Vergleichende Untersuchungen über die Einfriertechnik des Bullensamens mit Hilfe der Paillette bzw. Ampulle. *Dt. tierärztl. Wschr.* **76**, 346.

Krause, D., and Grove, D. (1967).—Deep-freezing of jackass and stallion semen in concentrated pellet form. *J. Reprod. Fert.* **14**, 139.

Lightfoot, R. J., and Salamon, S. (1969*a*).—Freezing ram spermatozoa by the pellet method. II. The effects of method of dilution, dilution rate, glycerol concentration, and duration of storage at 5°C prior to freezing on survival of spermatozoa. *Aust. J. biol. Sci.* **22**, 1547.

Lightfoot, R. J., and Salamon, S. (1969*b*).—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. *Aust. J. biol. Sci.* **22**, 1561.

Nagase, H., and Niwa, T. (1963).—Studies on deep freezing technique for bull semen. III. Deep freezing of bull semen in tablet form. *Jap. J. anim. Prod.* **9**, 93.

Nagase, H., and Niwa, T. (1964).—Deep freezing bull semen in concentrated pellet form. I. Factors affecting survival of spermatozoa. Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 410.

Platov, E. M. (1965).—Freezing of ram semen in egg yolk–lactose–ethylene glycol diluent. *Ovtsevodstvo* **11**(9), 11.

ROHLOFF, D. (1967).—Tiefgefrierung von Ebersperma in Pelletform bei −196°C. *Zuchthygiene* **2**, 75.

SALAMON, S. (1968).—Deep freezing of ram semen: recovery of spermatozoa after pelleting and comparison with other methods of freezing. *Aust. J. biol. Sci.* **21**, 351.

SALAMON, S. (1971).—Fertility of ram spermatozoa following pellet freezing on dry ice at −79 and −140°C. *Aust. J. biol. Sci.* **24**, 183.

SALAMON, S., and BRANDON, M. R. (1971).—Effect of composition of the thawing solution on survival of ram spermatozoa frozen by the pellet method. *Aust. J. biol. Sci.* **24**, 355.

SAMOUILIDIS, S. (1970).—Ein Beitrag zur Tiefkühlkonservierung von Schaf-und Ziegenbocksamen mit Hilfe des Pellet-bzw. Paillettenverfahrens. Vet.-med. Dissertation, Tierärztl. Fak. Ludwig-Maximilians-Univ., München. [*Anim. Breed Abstr.* **39**, No. 1408 (1971).]

STEINBACH, J., and FOOTE, R. H. (1967).—Osmotic pressure and pH effects on survival of frozen bovine spermatozoa. *J. Dairy Sci.* **50**, 205.

YASSEN, A. M., and FOOTE, R. H. (1967).—Freezability of bovine spermatozoa in Tris-buffered yolk extenders containing different levels of Tris, sodium, potassium, and calcium ions. *J. Dairy Sci.* **50**, 887.