DEEP FREEZING OF RAM SEMEN: RECOVERY OF SPERMATOZOA AFTER PELLETING AND COMPARISON WITH OTHER METHODS OF FREEZING

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Summary

Four factorial experiments were conducted in which were examined and compared factors which affect revival of ram spermatozoa after pellet-freezing and other methods of freezing.

Revival of spermatozoa after pellet-freezing was the best in egg yolk–lactose (333 mM), followed by egg yolk–raffinose (333 mM), egg yolk–glucose (355 mM), and egg yolk–fructose (355 mM) diluents. Within the glycerol range of 0–7%, lactose gave the best results with 3%, raffinose and glucose with 5%, and fructose with 7% (v/v) glycerol.

Thawing solutions of 2·6 or 3·1% sodium citrate gave similar revival rates. Thawing of pellets at 45, 40, or 30°C was superior to thawing at 20, 15, 10, or 5°C, and revival was improved by using increasing amounts of thawing solution.

Centrifugation of egg yolk–lactose, egg yolk–raffinose, and egg yolk–fructose diluents or replacement of 100 mM of the sugars by equimolar amounts of sodium citrate improved revival rates. Diluents and step of dilution [i.e. addition of glycerol shortly after semen collection at 30°C (one-step dilution) or after cooling at 5°C (two-step dilution)] interacted. While glucose gave poorer survival after one-step than after two-step dilution, both lactose and raffinose yielded best results with the one-step method. Equilibration for 2·5 hr was better than no equilibration, and the effect was more beneficial when glycerol was added after cooling to 5°C (two-step dilution).

There were no significant differences in revival rates after either pelleting diluted semen on dry ice or freezing in ampoules at a slow rate, and both methods were superior to freezing in synthetic straws. Rapid freezing of semen in ampoules to −79°C gave poor recovery, and only a few spermatozoa (8·8%) survived after pelleting directly into liquid nitrogen.

Glycerol (5%) gave better protection to spermatozoa than did ethylene glycol (5%) or combination of either glycerol (3%) or ethylene glycol (3%) with dimethyl sulphoxide (2%). Dimethyl sulphoxide (5%) resulted in very poor revival.

The semen of the five rams used differed significantly in behaviour on freezing.

I. INTRODUCTION

Successful freezing of bovine semen in pellet form by Nagase and Niwa (1963, 1964), and the subsequent acceptable fertility obtained by these (Nagase and Graham 1964; Nagase, Graham, and Niwa 1964; Nagase *et al.* 1964*a*) and other investigators (Barrière 1965; Parez 1965; Rajamannan 1965; Ström 1965) has stimulated attempts to freeze ram semen by this method (Platov 1965; Platov and Sevcova 1966; Salamon and Lightfoot 1967).

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A fertility trial with pellet-frozen ram semen, using an egg yolk–citrate–glucose diluent, gave disappointing results (Salamon 1967), and a number of factors which could influence revival of ram spermatozoa after pellet-freezing remained unclarified. This is a report of experiments undertaken to investigate some of these factors.

II. Material and Methods

Four factorial experiments were conducted and the comparisons made in each are shown in the following tabulation:

Expt. No.	Design	Factors
1	$4\times5\times2\times2$	Diluent × glycerol concentration × thawing solution × replicate
2	$7\times6\times2$	Thawing temperature × post-thawing dilution rate × glycerol concentration
3	$3\times2\times2\times2\times2\times4$	Diluent × absence or presence of citrate in the diluent × processing (centrifugation) of diluent × step of dilution × equilibration × incubation
4	$6\times5\times5$	Method of freezing × protective agent × rams

Semen was collected from five mature Merino rams by means of an artificial vagina. Ejaculates of high initial motility from individual rams were either pooled (expts. 1, 2, 3) or used on a split-sample basis (expt. 4).

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

The diluents used consisted of fructose and glucose, each in a final prefreezing concentration of 355 mM, and of lactose and raffinose, 333 mM each (expts. 1 and 2). When citrate was included in the diluents (expts. 3 and 4), 100 mM of the respective sugar was replaced by an equimolar amount of sodium citrate. All diluents contained 15% (v/v) fresh egg yolk.

Soon after collection the semen samples were diluted at 30°C with the non-glycerolated portion to half of the final dilution (two-step, expts. 1 and 2), or with the glycerol-containing diluent to final freezing dilution (one-step, expts. 3 and 4). In the case of the two-step dilution, the glycerol-containing fraction was added after cooling to 5°C. Both one- and two-step-diluted samples were cooled from 30 to 5°C over 1 hr, and equilibrated at this temperature for a 2·5-hr period. The final (prefreezing) dilution rate in all experiments was one part of semen to two parts of diluent. The semen (0·03 ml) was pelleted on dry ice (Nagase and Niwa 1963, 1964). The pellets were kept on dry ice for 3–4 min, then transferred into dry containers and stored in liquid nitrogen.

The presence of egg yolk in the sugar diluents caused some turbidity, and the undispersed yolk particles often made evaluation of motility difficult when the thawing of pellets was carried out in dry vials or in a small amount of thawing solution. To overcome this the diluents used in experiment 3 were either centrifuged at 3500 *g* for 30 min or had sodium citrate included.

Experiment 4 compared freezing in ampoules, in Cassou's synthetic straws, or in pellet form, using glycerol (5%), ethylene glycol (5%), dimethyl sulphoxide (5%), and a combination of the first two (3% each) with dimethyl sulphoxide (2%) as protective agents.

The sealed ampoules, at a temperature of 5°C and containing 1 ml diluted semen, were frozen in two ways, namely, by (1) placing them into an alcohol bath at −20°C, and using further freezing rates as indicated by Lopatko (1962), or (2) plunging them directly into a dry ice–alcohol mixture at −79°C. The semen in synthetic straws was frozen either in vapour 2–3 cm above the surface of liquid nitrogen, or by placing them onto the surface of a block of dry ice for 15 min.

All frozen samples were stored in liquid nitrogen (−196°C) for at least 72 hr before thawing for evaluation.

(b) Thawing of Semen

Frozen semen samples were thawed in a water-bath at 37°C, except in experiment 2 where different thawing temperatures were compared. In this experiment thawing temperatures of 45, 40, 30, and 20°C were maintained in the water-bath with an automatic temperature regulator,

TABLE 1

EXPERIMENT 1: EFFECT OF DILUENT, GLYCEROL CONCENTRATION, AND THAWING SOLUTION ON REVIVAL OF PELLET-FROZEN SPERMATOZOA

Two-step dilution, pooled ejaculates. Bracketed values do not differ significantly

Main Effects	Motility Score	Motile Spermatozoa (%)
Diluent (n = 20)		
Egg yolk–fructose	1·55	14·8
Egg yolk–glucose	1·90	18·9
Egg yolk–raffinose	2·08	23·8
Egg yolk–lactose	2·58	29·5
Significance of differences	$P<0·001$	$P<0·001$
Glycerol concn. (n = 16)		
0	1·28	6·6
1%	1·56	19·0
3%	2·50	27·4
5%	2·69	32·6
7%	2·12	26·7
Significance of differences		
Linear	$P<0·001$	$P<0·001$
Quadratic	$P<0·001$	$P<0·01$
Cubic	$P<0·001$	n.s.
Quartic	$P<0·01$	n.s.
Thawing solution (n = 40)		
2·6% sodium citrate	2·03	21·8
3·1% sodium citrate	2·00	21·2
Significance of differences	n.s.	n.s.
Replicate* (n = 40)		
1	2·16	23·2
2	1·93	19·9
Significance of differences	$P<0·001$	$P<0·001$

* Collections on separate days.

and temperatures of 15, 10, and 5°C by holding and constantly controlling water cooled to these temperatures in isolated containers. Two pellets were thawed in four drops of 2·6% sodium citrate, except in experiment 2 where the ratio of pellet to thawing solution differed.

When semen was frozen in ampoules or straws (expt. 4), the post-thawing concentration of spermatozoa was adjusted to that of thawed pellets with 2·6% sodium citrate solution.

(c) *Examination of Spermatozoa Recovery*

Motility (score 0–5) and percentage motile spermatozoa were evaluated under a coverslip on a warm stage (37°C) immediately after thawing and at 2-hr intervals when incubation at 37°C was carried out.

(d) *Analyses of Data*

Data for each experiment, following transformation of percentages to angles, were examined by analyses of variance. Results from experiments involving incubation (expt. 3) were analysed according to the method outlined by Snedecor (1956) for a split-plot experiment. Differences between means, where required, were tested by multiple-range test (Snedecor 1956). All percentages shown in the tables are reconverted values of the means for the transformed data.

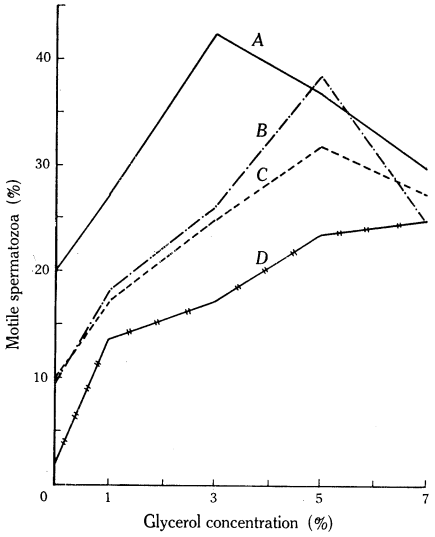

Fig. 1.—Interaction of diluents and glycerol concentration on the percentage motile spermatozoa after pellet-freezing.

A, Egg yolk–lactose.
B, Egg yolk–raffinose.
C, Egg yolk–glucose.
D, Egg yolk–fructose.

III. Results

(a) *Experiment* 1

The best revival of spermatozoa after pelleting was obtained with egg yolk–lactose, followed by egg yolk–raffinose diluent (Table 1). The multiple-range test revealed no significant difference between the mean percentages of motile spermatozoa when 3, 5, or 7% glycerol was used, although motility scores varied markedly. There were significant diluent × glycerol interactions ($P < 0{\cdot}001$) for both characteristics assessed, and that for percentage of motile spermatozoa is presented in Figure 1. Thawing media of 2·6 or 3·1% sodium citrate gave similar results.

(b) *Experiment* 2

The temperature of thawing had a marked effect on recovery of spermatozoa, the best results being obtained with thawing at 30 and 40°C and the poorest at 15°C (Table 2).

The ratio of pellet to thawing solution significantly affected revival rates, and there were declines for both motility score and percentage of motile spermatozoa with decreasing post-thawing dilution rates.

There was an interaction between temperature and thawing dilution rate for percentage of motile spermatozoa ($P < 0{\cdot}001$). Nevertheless, the highest values for all dilution rates were obtained by thawing at 30 and 40°C.

Glycerol in 3 or 5% concentration gave similar results.

TABLE 2

EXPERIMENT 2: EFFECT OF THAWING TEMPERATURE AND POST-THAWING DILUTION RATE ON REVIVAL OF PELLET-FROZEN SPERMATOZOA

Egg yolk–lactose diluent, two-step dilution, pooled ejaculates. Bracketed values do not differ significantly

Main Effects	Motility Score	Motile Spermatozoa (%)
Thawing temperature ($n = 12$)		
40°C	3·35	45·8
30°C	3·21	44·1
45°C	3·02	42·1
20°C	2·92	35·2
15°C	1·54	20·8
10°C	2·17	28·6
5°C	2·13	27·8
Significance of differences	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Dilution rate post-thawing ($n = 14$) (pellet : thawing solution)		
1 : 3	2·82	38·7
1 : 2	2·75	37·7
1 : 1	2·63	35·4
2 : 1	2·79	34·7
3 : 1	2·57	33·6
Thawing in dry vials (no thawing solution)	2·16	28·4
Significance of differences	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Glycerol concn. ($n = 42$)		
3%	2·67	36·0
5%	2·55	34·0
Significance of differences	n.s.	n.s.

(c) *Experiment* 3

Tables 3 and 4 show that fewer motile spermatozoa were detected after thawing and during subsequent incubation in glucose than in lactose and raffinose diluents.

The diluents containing citrate were slightly superior ($P < 0{\cdot}05$), and this was more evident after one-step dilution, as indicated by the citrate × step of dilution interaction ($P < 0{\cdot}01$).

A significantly higher revival rate was observed in centrifuged than in uncentrifuged diluent, and the decline in survival during the 6-hr incubation was steeper in the uncentrifuged diluent (processing of diluent × incubation, $P < 0{\cdot}05$).

TABLE 3

EXPERIMENT 3: EFFECT OF DILUENT, PROCESSING OF DILUENT BEFORE FREEZING, STEP OF DILUTION, EQUILIBRATION, AND INCUBATION TIME ON SURVIVAL OF PELLET-FROZEN SPERMATOZOA DURING INCUBATION AT 37°C

3% glycerol, pooled ejaculates

Main Effects	Motility Score	Motile Spermatozoa (%)
Diluent ($n = 64$)		
Egg yolk–glucose	2·55	19·7
Egg yolk–lactose	2·80	30·8
Egg yolk–raffinose	2·73	30·6
Significance of differences	$P < 0{\cdot}01$	$P < 0{\cdot}001$
Sodium citrate in diluent ($n = 96$)		
Absent	2·68	25·3
Present	2·71	28·4
Significance of differences	n.s.	$P < 0{\cdot}05$
Processing of diluent ($n = 96$)		
Centrifuged	2·91	32·6
Not centrifuged	2·48	21·5
Significance of differences	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Step of dilution ($n = 96$)		
One-step	2·68	26·2
Two-step	2·71	27·5
Significance of differences	n.s.	n.s.
Equilibration time ($n = 96$)		
0 hr	2·63	23·9
2·5 hr	2·77	29·9
Significance of differences	$P < 0{\cdot}05$	$P < 0{\cdot}001$
Incubation time ($n = 48$)		
0 hr	2·98	35·5
2 hr	2·82	32·7
4 hr	2·57	22·4
6 hr	2·42	18·2
Significance of differences		
Linear	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Quadratic	n.s.	n.s.
Cubic	n.s.	$P < 0{\cdot}001$

The main effects for step of dilution were indistinguishable, but there was a diluent × step of dilution interaction ($P < 0{\cdot}001$) showing that, while glucose diluent gave poorer survival following one-step than after two-step dilution, both lactose and

raffinose yielded best results with the one-step method (see following tabulation):

Diluent	Motile Spermatozoa (%) One-step Dilution	Two-step Dilution
Egg yolk–glucose	14·7	25·1
Egg yolk–lactose	32·5	29·2
Egg yolk–raffinose	33·0	28·3

Equilibration period of 2·5 hr increased both motility and percentage motile spermatozoa (from 24·9 to 27·6% for one-step dilution, and from 23·0 to 32·3% for two-step dilution) and the effect (significant at $P<0·05$) was more beneficial when glycerol was added after cooling to 5°C (interaction significant at $P<0·05$).

TABLE 4

EXPERIMENT 3: ANALYSES OF VARIANCE

Source of Variation	Degrees of Freedom	Variance Ratios Motility Score	Motile Spermatozoa (%)
Diluents (*A*)	(2)	10·98**	24·44***
Glucose *v.* lactose and raffinose	1	21·04***	48·87***
Lactose *v.* raffinose	1	0·92	0·01
Absence *v.* presence of citrate (*B*)	1	0·45	4·85*
Step of dilution (*C*)	1	0·31	0·72
Equilibration (*D*)	1	9·12*	15·56***
Processing of diluent (*E*)	1	84·10***	52·42***
First-order interactions:			
$A \times C$	2	3·61	11·47***
$B \times C$	1	5·52*	10·29**
$C \times D$	1	6·05*	4·92*
$C \times E$	1	10·52**	4·61
Remainder	9	1·50	0·82
Pooled second-order interactions	16	1·48	0·83
Error mean square (pooled third- and fourth-order interactions)	11	0·10	46·92
Incubation (*F*):	(3)		
Linear	1	116·65***	308·73***
Quadratic	1	0·02	1·72
Cubic	1	0·85	13·64***
Interactions:			
$E \times F$	3	4·24*	5·25*
Remainder	105	0·93	0·98
Error mean square (pooled fourth- and fifth-order interactions)	33	0·08	12·84

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

(*d*) *Experiment* 4

In this experiment, ejaculates from individual rams were frozen by different methods on a split-ejaculate basis (Table 5). The best results were obtained after pelleting on dry ice and freezing in ampoules at a slow rate. Freezing of synthetic

straws in liquid nitrogen vapour was superior to freezing on dry ice. Recovery was poor when ampoules were plunged into a dry ice–alcohol bath at −79°C, and only a few spermatozoa were motile after dropping diluted semen directly into liquid nitrogen.

Glycerol (5%) gave better protection to spermatozoa during freezing than ethylene glycol (5%) or their combination with dimethyl sulphoxide. The use of dimethyl sulphoxide alone in 5% concentration resulted in very poor recovery.

TABLE 5

EXPERIMENT 4: EFFECT OF METHOD OF FREEZING AND PROTECTIVE AGENT ON REVIVAL OF SPERMATOZOA AFTER THAWING

Egg yolk–citrate–lactose diluent, one-step dilution. Bracketed values do not differ significantly

Main Effects	Motility Score	Motile Spermatozoa (%)
Method of freezing (n = 25)		
Pellets in liquid nitrogen	1·13	8·8
Pellets on dry ice	3·04	34·5
Ampoules slow freezing	2·96	32·9
Ampoules fast freezing (−79°C)	2·28	16·2
Straws on dry ice	2·48	20·8
Straws in liquid nitrogen vapour	2·70	26·9
Significance of differences	$P<0·001$	$P<0·001$
Protective agent (n = 30)		
Glycerol 5%	3·32	36·5
Ethylene glycol 5%	2·37	26·5
Glycerol 3%, dimethyl sulphoxide 2%	2·43	25·7
Ethylene glycol 3%, dimethyl sulphoxide 2%	2·35	18·4
Dimethyl sulphoxide 5%	1·69	7·7
Significance of differences	$P<0·001$	$P<0·001$
Rams (n = 30)		
Ram 3	2·85	31·2
Ram 1	2·69	27·3
Ram 4	2·40	22·6
Ram 2	2·23	18·2
Ram 5	1·97	15·1
Significance of differences	$P<0·001$	$P<0·001$

There were interactions of method of freezing and protective agent for both motility score and percentage of motile spermatozoa ($P<0·001$). Nevertheless, the best recovery by each method of freezing was obtained when glycerol was used.

The semen of the five rams differed significantly in their behaviour on freezing and, although there was a ram × protective agent interaction on percentage of motile spermatozoa ($P<0·05$), glycerol gave the best protection for semen of all rams.

IV. Discussion

Lactose and raffinose proved to be better components of diluents for the fast freezing of ram semen than were fructose and glucose. These two groups of sugar diluents had different concentrations, but it is unlikely that this substantially affected the results, because in further unpublished pellet-freezing studies, using sugar diluents in varying concentrations, glucose at any level yielded poorer recovery than either lactose or raffinose.

Differences in values of the limited number of sugars tested here seem to support the view of Nagase *et al.* (1964*b*) that sugars of higher molecular weight provide better protection to spermatozoa during fast freezing than do those of lower molecular weight. The effect of sugars in the diluent used for freezing ram semen by conventional methods has been reported by Emmens and Blackshaw (1950) and Jones and Martin (1965), and various sugars for chilling have been examined by Martin (1966) and Lapwood and Martin (1966).

Inclusion of citrate in the sugar diluent, or previous centrifugation, could facilitate assessment of motility of pellet-frozen spermatozoa if no thawing solution were used. The presence of citrate did not reduce viability of spermatozoa, contrary to observations of Jones and Martin (1965). It is unlikely that centrifugation or presence of citrate in the diluent simply provided a more accurate assessment, since clear fields were visible under the microscope for all untreated egg yolk–sugar diluents after three-fold post-thawing dilution of pellets with citrate solution.

The glycerol level in the sugar diluent used for pelleting ram spermatozoa, as for the bull (Nagase, Graham, and Niwa 1964; Nagase *et al.* 1964*a*), can be decreased to 3%, but the rate of reduction seems to depend upon which sugar is used. Lactose can give reasonable survival with only 1% glycerol, or even without glycerol. The sugar used appears to determine also whether the glycerol should be added at 30°C (one-step) or after cooling to 5°C (two-step), but an equilibration period of 2·5 hr appears beneficial following both, especially after the two-step dilution method.

Ethylene glycol in 5% concentration was inferior to glycerol (5%) as a protective agent. Platov (1965), however, reported that 1·75 and 2% ethylene glycol gave protection to ram spermatozoa during pellet-freezing, similar to that of 3·5% glycerol. Dimethyl sulphoxide proved to be toxic to ram spermatozoa, so confirming the results of Jones (1965*a*, 1965*b*).

There are numerous reports on the effect of thawing temperature on revival of spermatozoa after conventional freezing, but there is no agreement among investigators (reviewed by Picket *et al.* 1965). The results presented here indicate that rapid thawing is required in order to obtain maximum recovery of spermatozoa after fast freezing. The improvement in recovery when larger amounts of thawing solutions are used could be attributed to a more rapid warming and thawing process. For insemination trials, however, low or no post-thawing dilution would be preferred in order to hold a high concentration of spermatozoa in the inseminating dose.

Freezing in synthetic straws, reported to give good results with bull semen (Cassou 1964; Jondet 1964; Macpherson and King 1966), was less effective with ram semen than was freezing in ampoules at a slow rate or in pellet form. A fertility trial,

however, showed no difference in lambing rates when semen frozen by these three methods was used for insemination (Salamon 1967). Rapid freezing in ampoules (−79°C), contrary to observations of Kalev and Venkov (1961), gave poor recovery. Finally, pelleting directly into liquid nitrogen was unsatisfactory. There remains the possibility that a temperature range between −120° and −140°C could be more effective for "ultra-rapid" freezing, as indicated by Goossens and Kamps (1966).

The large differences in freezability of ejaculates from individual rams shows the necessity for careful selection of samples for freezing, and semen from particular rams could require different methods of processing for satisfactory storage and revival.

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VI. References

Barrière, J. (1965).—*Élevage Insem.* **88**, 9.
Cassou, R. (1964).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4, p. 540.
Emmens, C. W., and Blackshaw, A. W. (1950).—*Aust. vet. J.* **26**, 226.
Goossens, J. M. M., and Kamps, W. J. G. (1966).—*Tijdschr. Diergeneesk.* **91**, 723.
Jondet, R. (1964).—*Élevage Insem.* **84**, 3.
Jones, R. C. (1965*a*).—*Aust. J. biol. Sci.* **18**, 877.
Jones, R. C. (1965*b*).—*Aust. J. biol. Sci.* **18**, 887.
Jones, R. C., and Martin, I. C. A. (1965).—*J. Reprod. Fert.* **10**, 413.
Kalev, G., and Venkov, T. (1961).—Proc. 4th Int. Congr. Anim. Reprod., The Hague. Vol. 4. p. 972.
Lapwood, K. R., and Martin, I. C. A. (1966).—*Aust. J. biol. Sci.* **19**, 655.
Lopatko, M. I. (1962).—*Zivotnovodstvo* **24**(10), 86.
Macpherson, J. W., and King, G. J. (1966).—*Can. J. comp. Med.* **30**, 109.
Martin, I. C. A. (1966).—*Aust. J. biol. Sci.* **19**, 645.
Nagase, H., and Graham, E. F. (1964).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 387.
Nagase, H., Graham, E. F., and Niwa, T. (1964).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 404.
Nagase, H., and Niwa, T. (1963).—*Jap. J. anim. Reprod.* **9**, 93.
Nagase, H., and Niwa, T. (1964).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 410.
Nagase, H., Niwa, T., Yamashita, S., and Irie, S. (1964*a*).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 503.
Nagase, H., Niwa, T., Yamashita, S., and Irie, S. (1964*b*).—Proc. 5th Int. Congr. Anim. Reprod., Trento. Vol. 4. p. 498.
Parez, M. (1965).—*Élevage Insem.* **88**, 3.
Picket, B. W., Hall, R. C. Jr., Lucas, J. J., and Gibson, E. W. (1965).—*Fert. Steril.* **16**, 642.
Platov, E. M. (1965).—*Ovcevodstvo* **11**(9), 11.
Platov, E. M., and Sevcova, A. A. (1966).—*Ovcevodstvo* **12**(9), 26.
Rajamannan, A. H. J. (1965).—*J. Dairy Sci.* **48**, 807.
Salamon, S. (1967).—*Aust. J. exp. Agric. Anim. Husb.* **7**, 559.
Salamon, S., and Lightfoot, R. J. (1967).—*Aust. J. agric. Res.* **18**, 959.
Snedecor, G. W. (1956).—"Statistical Methods." 5th Ed. (Iowa State College Press: Ames, Iowa.)
Ström, B. (1965).—*Svensk Husdjurssköts el* **9**, 268.